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13. ABSTRACT *(Maximum 200 Words)*

The goal of this investigation is to determine the three-dimensional structures of the two known human estrogen receptors (ERα and ERβ) complexed with receptor-selective estrogens and antiestrogens (SERMs). The crystallographic structures of ERα and ERβ ligand binding domains complexed with *cis*-R,R-diethyl-tetrahydrochrysene-2,8-diol (R,R-THC) have been solved and refined, suggesting mechanisms by which this compound can act as an ERα agonist and as an ERβ antagonist. Agonists and antagonists bind at the same site within the core of the ER LBD to induce distinct conformations in the transactivation domain (AF-2), especially in the positioning of helix 12. Previously determined structures of ERα with 4-hydroxytamoxifen (OHT) and diethylstilbestrol (DES) revealed and defined a multipurpose docking site on ERα and ERβ that can accommodate either helix 12, in the presence of OHT, or one of several co-regulators in the presence of DES. R,R-THC stabilizes a conformation of the ERα LBD that favors coactivator association and a conformation of the ERβ LBD that prevents coactivator association. A comparison of the two structures, combined with functional data, reveals that THC does not act on ERβ through the same mechanisms used by other known ER antagonists. Instead, THC antagonizes ERβ through a novel mechanism we term "passive antagonism". Paradoxically, the R,R-THC-ERβ structure is very similar to the structure induced by genistein, which acts as a partial estrogen through both ER subtypes. Ongoing mutagenesis studies should help define the molecular and structural differences that are responsible for these unanticipated results. The passive antagonism mechanism suggests a novel approach to the design of ligands that selectively antagonize the two ER subtypes. Such ligands may have novel therapeutic properties that can be exploited to prevent or treat breast cancer.

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INTRODUCTION

The goal of this investigation is to determine the three-dimensional structures of the two known human estrogen receptors (ERα and ERβ) complexed with receptor-selective estrogens and antiestrogens (SERMs). An important, unresolved issue is the molecular mechanism(s) by which SERMs can act selectively as full agonists, partial agonists, or complete antagonists in the control of cell proliferation and cell fate in different tissues. The physiological effects of both endogenous and synthetic SERMs are mediated by the estrogen receptors (ERs), ERα and ERβ, members of the nuclear receptor (NR) super-family of ligand-regulated transcription factors. The two ER subtypes have overlapping but distinct tissue distribution patterns *in vivo* and distinct activation profiles at both classical and complex promoter elements. Thus, it is unclear how ERα and ERβ individually contribute to the effects of known estrogens. Ligands that act differentially on ERα and ERβ would be potentially valuable both as tools to dissect the biological roles of the two ERs and as novel therapeutics with pharmacological properties distinct from existing drugs. As part of a search for ER subtype-selective ligands, the synthetic compound, R,R-5,11-*cis*-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (R,R-THC), was identified as a selective estrogen agonist when bound to ERα and as an antagonist when bound to ERβ. To better understand this selective behavior, a major goal of this investigation was to determine the crystallographic structures of human ERα and ERβ ligand binding domains (LBDs) complexed with R,R-THC. These structures have now been solved and refined, suggesting mechanisms by which this compound can act as an ERα agonist and as an ERβ antagonist. Previously determined structures of ERα with 4-hydroxytamoxifen (OHT) and diethylstilbestrol (DES) (1) revealed and defined a multipurpose docking site on ERα and ERβ that can accommodate either helix 12, in the presence of OHT, or one of several co-regulators in the presence of DES. Thus, agonists and antagonists bind at the same site within the core of the ER LBD to induce distinct conformations in the transactivation domain (AF-2), especially in the positioning of helix 12. Interestingly, R,R-THC stabilizes a conformation of the ERα LBD that favors coactivator association and a conformation of the ERβ LBD that prevents coactivator association. A comparison of the two structures, combined with functional data, reveals that R,R-THC does not act on ERβ through the same mechanisms used by other known ER antagonists. Instead, R,R-THC antagonizes ERβ through a novel mechanism we term "passive antagonism". Paradoxically, the R,R-THC-ERβ structure is very similar to the structure induced by genistein (2), which acts as a partial estrogen through both ER subtypes. Ongoing mutagenesis studies should help define the molecular and structural differences that are responsible for these unanticipated results. In addition, the passive antagonism mechanism suggests a novel approach to the design of ligands that selectively antagonize the two ER subtypes. Such ligands may have novel therapeutic properties that can be exploited to prevent or treat breast cancer.

BODY

The Statement of Work for this grant listed four major tasks. These tasks are listed below with details of the progress made toward achieving each goal.

Task 1 (First year): To produce and purify bacterially expressed ERα-LBD-BJK-1 complexes for biochemical analysis and crystallization.

As described in detail in the attached manuscript (see Appendix) and in last year's Annual Report, this task has been accomplished. BJK-1 is the same as R,R-THC. We did not know the identity of BJK-1 at the time the proposal was submitted to the USAMRMC in 1998.

Task 2 (Second year): Purification, crystallization, and x-ray analysis of ERα/β-LBD-BJK-1 and ERα/β-LBD-ICI complexes.

The major portion of this task has been accomplished, as described in the attached manuscript (see Appendix) and in last year's Annual Report. We did not pursue either the ERα-or ERβ-ICI 182,780 complexes because the R,R-THC studies consumed all available personnel time for this grant. During this period, we learned that the Hubbard lab had solved the structure of rat ERβ LBD complexed with ICI 164,384; the structure was published earlier this year (3). Because this structure is expected to be identical to the human ERβ LBD complexed with ICI 182,780 and very similar to the ERα LBD complex, we abandoned this aim, although it may still be of some value to know if the ERα-ICI conformation is different from the ERβ-ICI conformation. Noteworthy in the ICI-ERβ structure was the absence of any association of the transactivation helix (H12) with the LBD, which contrasts with all other known antagonist-ER structures, in which H12 is associated with the hydrophobic cleft formed by helices 3, 4 and 5 (**Appendix, Figure 2A**). In the ICI-ERβ complex, the terminal portion of the ICI bulky side chain substituent emerges from the ligand-binding pocket and binds to part of the coactivator recruitment site (AF2), thereby preventing H12 from adopting either its characteristic agonist or antagonist orientation (**Figure 1**). This complete destabilization of H12 may contribute to the more potent antiestrogenic activity of both closely related ICI compounds when compared to SERMs like tamoxifen and raloxifene. Another potentially significant difference is the effect of the ICI 164,384 on the ERβ dimerization interface. For all other known ERα/β LBD structures, the ligand has little effect on the nature or extent of the dimer interface. However, ICI appears to generate a more open and perhaps less stable dimer, consistent with reports that ERα-ICI dimers are less stable than other ligand-ER complexes (4). It is also possible that a crystallization artifact is responsible for the observed ICI-induced conformation, or that the ERβ dimer interface is more sensitive than ERα to ligand effects (3). For ERβ-R,R-THC, the dimer interface is very similar to the ERβ-Ral and ERβ-Gen structures (2, 3).

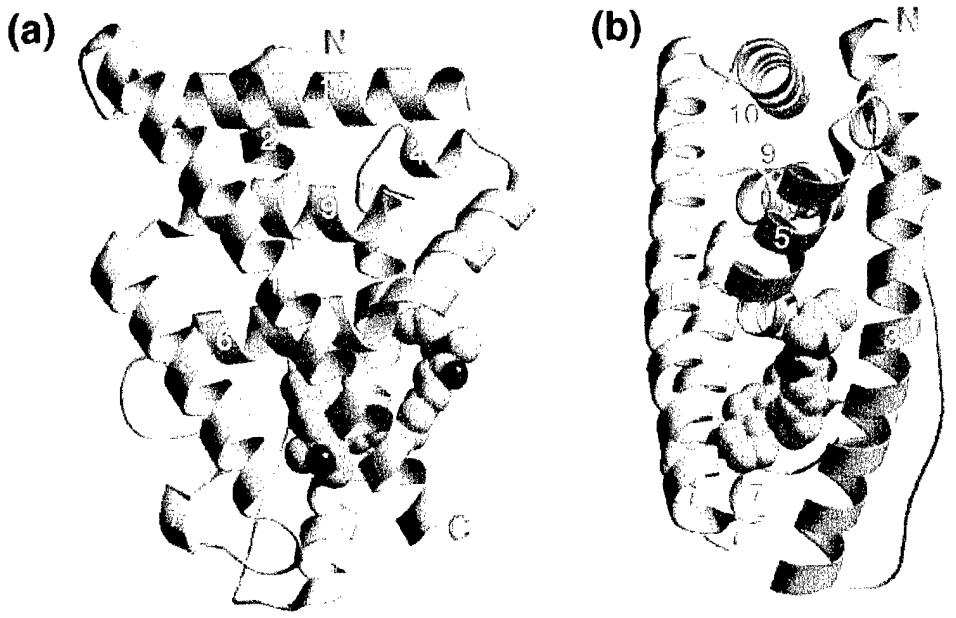

Figure 1. Structure (orthogonal views) of ERβ-LBD bound to the pure antagonist ICI 164,384. Reproduced from Pike et al. (3).

Analysis of protein structure is always a work in progress and the R,R-THC-ERα/β LBD structures are no exception. Although we were able to obtain preliminary crystallographic solutions for both the R,R-THC-ERα and R,R-THC-ERβ LBD structures more than a year ago, ongoing data refinement during the past year revealed a more complex ligand interaction with the ERβ pocket than proposed initially. As shown in **Figure 2**, our original assumption that fewer contacts between R,R-THC and the amino acid residues that line the pocket of ERβ were responsible for the inability of this complex to assume an agonist conformation was incorrect. Instead, an equal or greater number of contacts occur between R,R-THC and amino acids that comprise the ligand-binding pocket, as compared to the R,R-THC-ERα LBD complex (**Appendix, Figure 4B**). The ERα complex structure has now been refined to a crystallographic R factor of 20.3% (R_{free} = 24.2%) using data to 1.95 Å resolution and the ERβ complex structure has been refined to a crystallographic R factor of 26.5% (R_{free} = 30.4%) using data to 2.85 Å resolution (**Appendix, Table 1**). It proved especially difficult to obtain an R_{free} value of 30.4% for ERβ, which is why we did not initially see the additional contacts that were present between R,R-THC and the ERβ LBD pocket. For example, we did not initially see the additional hydrogen bond that occurs between the A ring phenolic OH of R,R-THC and Arg 346, similar to the interaction with Arg 394 in the R,R-THC-ERα structure (**Figure 2B; Appendix, Figure 4B**). Similarly, additional R,R-THC contacts with the ERβ LBD pocket could only be appreciated

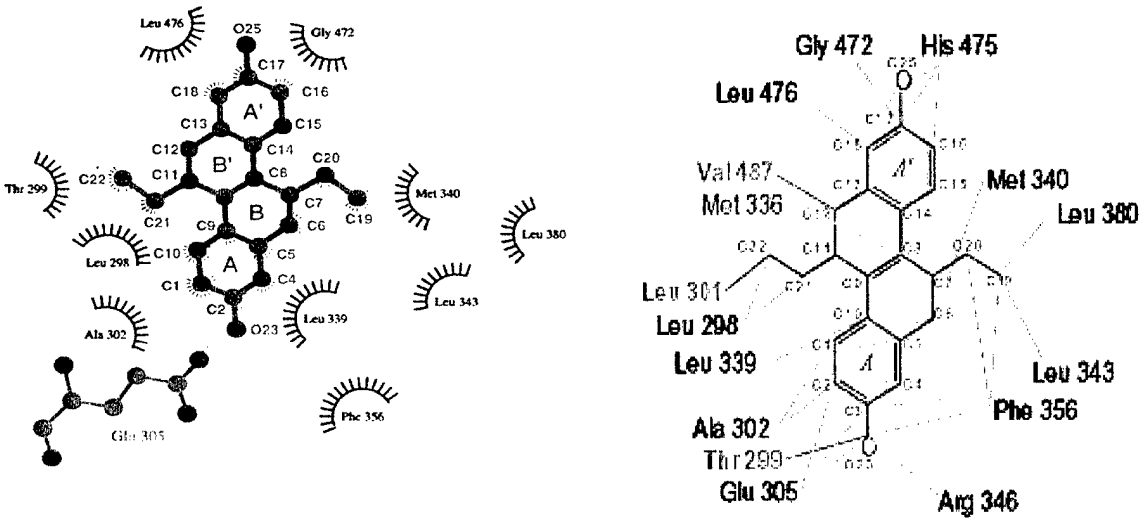

Figure 2. R,R-THC Interactions with ERβ LBD Pocket. **A.** Previous model. **B.** Current model based on refined structure (see **Appendix, Figure 4B**).

upon further refinement of the structural data. We now feel that there are some 16 amino acids, rather than the 11 first observed (**Figure 2B; Appendix, Figure 4B**), that participate in the stabilization of R,R-THC in ERβ, which equals the 16 amino acids that are involved in the R,R-THC-ERα LBD complex, although not all residues are the same (**Appendix, Figure 4B**).

Thus, although our hypothesis of "passive antagonism" has not changed, our view of the underlying mechanism(s) has evolved. As described previously, the ERβ LBD shares the same overall fold as the ERα LBD (**Appendix, Figure 2A**) (2). And like the ERα LBD, the ERβ LBD recognizes R,R-THC with a ligand-binding pocket located within its lower sub domain.

However, the binding of R,R-THC to the ERβ LBD does not stabilize the agonist-bound conformation of helix 12 (**Appendix, Figure 2A**). Neither is helix 12 bound to the portion of the coactivator recognition groove formed by helices 3, 4, and 5 as it is in the OHT-ERα LBD (1), RAL-ERα LBD (5), and RAL-ERβ LBD (2) complexes (**Appendix, Figure 2B**), nor is it disordered as it is in the ICI-ERβ LBD complex (**Figure 1**) (3). Instead, helix 12 in the R,R-THC-ERβ LBD complex interacts with the rest of the LBD in a manner most like that observed in the GEN-ERβ LBD complex (**Appendix, Figure 2B**) (2). Helix 12 is positioned similarly, and of comparable length (residues 487 to 498 in the R,R-THC complex and residues 487 to 497 in the GEN complex) in the two structures (**Appendix, Figure 2B**).

Based on previous correlations between the positioning of H12 and the ability of ERα and other NRs to form coactivator complexes, the conformation of the ERβ LBD observed in both the R,R-THC and GEN complexes should be incapable of interacting with coactivators and, hence, should be transcriptionally silent for two reasons. First, key residues from helix 12 (Leu 490, Glu 493, and Met 494) that are predicted to form part of the coactivator recognition surface are inappropriately positioned. In addition, helix 12 itself is bound such that it partially occludes the static region of the coactivator-binding surface formed by residues from helices 3, 4, and 5. Yet, GEN and R,R-THC clearly exhibit different activities on ERβ in mammalian cells; GEN acts as a partial agonist (6) and R,R-THC acts as a pure antagonist (7, 8). How can the similarity of the two structures be reconciled with the different activities of these compounds in transcriptional assays? The simplest model that explains these and other data is based on two suppositions (**Appendix, Figure 3A**). First, helix 12 in the unliganded ERβ LBD is in equilibrium between the R,R-THC/GEN-bound conformation, which precludes coactivator association, and the agonist-bound conformation seen in the E2- and DES-ERα LBD structures, which favors coactivator association (with the "inactive" R,R-THC/GEN-bound conformation being heavily favored). Second, rather than stabilizing a single static conformation of helix 12, ligands serve to shift the balance of this equilibrium. It is also highly likely that the coactivator receptor-interaction domain (e.g. GRIP NR box II domain) plays an active role in shifting this equilibrium.

R,R-THC could antagonize receptor activity by shifting equilibrium in favor of the inactive R,R-THC/GEN-bound conformation (**Appendix, Figure 3A**). If R,R-THC binding forces the receptor to favor this conformation even more than it does in the absence of ligand, the R,R-THC-receptor complex would be expected to have an even lower affinity for coactivator than the unliganded receptor. This would be consistent with the absence of transcriptional activity of the R,R-THC-ERβ complex and the conformation of helix 12 observed in the crystal.
Because the conformational equilibrium of helix 12 would be difficult to observe directly, we chose to test the hypothesis that the binding of different ligands should modulate the affinity of the ERβ LBD for coactivator such that E2-LBD ~ DES-LBD > GEN-LBD > apo-LBD > R,R-THC-LBD. The affinities of various ligand-ERβ LBD complexes for an LXXLL motif-containing peptide were therefore directly measured using a fluorescence polarization-based binding assay (**Appendix, Figure 3B**) (9). The full agonist complexes bind the peptide approximately 1.5-fold more tightly than the GEN complex (K_d[E2] = 0.36 μM and K_d[DES] = 0.41 μM versus K_d[GEN] = 0.58 μM). In addition, the GEN complex binds the peptide more tightly than the unliganded receptor (K_d[apo] = 2.1 μM), whereas the R,R-THC complex (K_d[R,R-THC] = 3.7 μM) binds the peptide significantly weaker than the unliganded receptor.

Thus, these data are entirely consistent with the described helix 12 conformational equilibrium model. Furthermore, recent mutational and crystallographic data suggest that the positioning of helix 12 in ERα (10) and other NRs (11) is also dictated by a ligand-sensitive conformational equilibrium.

To better define and understand "passive antagonism", or antagonism without side chains, one has to consider the differences between classical estrogen antagonists, which contain bulky side chains, and molecules like R,R-THC. Although agonists and antagonists bind at the same site within the core of the LBD, each induces distinct conformations in the transactivation domain (AF-2) of the LBD, especially in the positioning of helix 12, providing structural evidence for multiple mechanisms of selective antagonism in the nuclear receptor family. Interestingly, the OHT/RAL and DES/E2 structures collectively reveal and define a multipurpose docking site on ERα that can accommodate either helix 12 or one of several coregulators. In addition, a comparison of the two types of structures reveals that there are at least two distinct mechanisms by which structural features of OHT promote an inhibitory conformation of helix 12 (1, 5). Helix 12 positioning is determined both by steric considerations, such as the presence of an extended side chain in the ligand, and by local structural distortions in and around the ligand binding pocket. Thus, one would predict that effective estrogen antagonists do not necessarily require bulky or extended side chains.

The positioning of the side chains of OHT, RAL, and ICI directly or "actively" preclude the agonist-bound conformation of helix 12 by steric hindrance. Hence, we define their common mechanism of action as "active antagonism". Clearly, R,R-THC does not have a bulky side chain (**Appendix, Figure 1A**), and in its complex with ERβ, helix 12 is not sterically precluded from adopting the agonist-bound conformation as it is in the other antagonist complexes (**Appendix, Figures 2A and 4C**). Instead, R,R-THC antagonizes ERβ by stabilizing key ligand binding pocket residues in noninteracting conformations, and disfavoring the equilibrium to the agonist-bound conformation of helix 12. Thus, we term the mechanism of antagonism of R,R-THC as "passive antagonism". Passive antagonism may not be unique to R,R-THC and ERβ. There are many examples of NR ligands, which act as antagonists even though they are smaller than the endogenous agonists of these NRs. The synthetic androgen receptor antagonist, flutamide, is comparable in size to testosterone, and does not possess an obvious moiety that would act as an antagonist side chain (12).

Many NRs have multiple subtypes, that possess distinct expression patterns and that regulate distinct target genes. Antagonists generated through the addition of bulky side chains to agonist scaffolds are limited to being antagonistic on one or more subtypes of a particular NR. The passive antagonism mechanism, as revealed here through direct comparison of the two R,R-THC-ER LBD complexes, suggests a new approach to achieving NR antagonism. Compounds could be designed to selectively stabilize the inactive conformations of certain NR subtypes and the active conformations of others. Such ligands may exert novel biological and therapeutic effects.

A manuscript describing the above data was recently submitted to *Nature Structural Biology* for review (included in the Appendix). Clearly, significant progress was made during the past year

in our effort to understand the unique R,R-THC-induced conformation of ERβ that contributes to "passive antagonism". More on this subject is included in the summary of Tasks 3 & 4 below.

Tasks 3 & 4 (Third year): To compare and correlate structural differences among hERα/β-LBD complexes with the biological activity of hERα/β.

Work on these tasks has already begun and we have made significant progress in trying to understand the contributions of residues that line the ligand-binding pocket to ligand discrimination between ERα and ERβ. An effort to crystallize ERα and/or ERβ complexed with GW5638 is also under way in the lab, but the main effort related to this grant during the coming year will be to better understand what distinguishes ERα from ERβ in their ability to differentially recognize diverse ligands with unique behavior. We will also begin modeling ligands that should have predictable agonist or antagonist properties as we learn more about the similarities and differences between the two ER subtypes from our own structures and those reported by others in the field. In addition, we will continue to create and test the ability of mutant ERs to interact with known coactivators and/or corepressors and to act as selective transcription modulators in co-transfection assays.

As described above, R,R-THC does not behave as an ERβ agonist. Instead, it binds to ERβ without interacting with several residues that it contacts in ERα, and by forming alternative hydrogen bonds and additional packing contacts not observed in the ERα complex (**Appendix, Figures 4B and 4C**). Hence, R,R-THC is able to bind to the ERβ LBD without nucleating many of the equivalent cooperative interactions observed in the ERα complex. For example, in the ERα complex the side chain of Met 528 packs against the side chains of His 524 and Met 343, stabilizing the helical conformation of the flanking residues. Because R,R-THC binding favors alternative conformations of His 475 and Met 295 (equivalent to His 524 and Met 343, respectively), the side chain of Met 479 (equivalent to Met 528) is completely disordered in the ERβ complex. In fact, by stabilizing conformations of binding pocket residues that prevent their interaction, R,R-THC binding should actually disfavor the equilibrium to the agonist-bound conformation of helix 12 (**Appendix, Figures 3A and 3B**).

A key unanswered question relates to the molecular/structural differences between the two ER subtypes that allow R,R-THC to stabilize these distinct arrangements of ligand binding pocket residues. There are only two binding pocket residues which are not identical in the two subtypes: Leu 384 (from helix 5/6) and Met 421 (from helix 8) in ERα, which are equivalent to Met 336 and Ile 373, respectively, in ERβ (**Figure 3**) (13, 14). These two residue pairs are similarly positioned on opposite faces of R,R-THC in their respective complexes. In the ERα complex, Leu 384 and Met 421 form several nonpolar contacts with R,R-THC (**Appendix, Figures 4B and 4C**). By contrast, only Met 336 interacts with R,R-THC in the ERβ complex (**Appendix, Figures 4B and 4C**).

Modeling studies suggest that Met 336 and Ile 373 would adopt both different positions and different conformations (relative to their positions in the R,R-THC-ERβ complex) in the agonist-bound conformation of the ERβ LBD (**Appendix, Figure 5**). There is currently no published structure of ERβ bound to a full agonist. However, given its similarity to the E2-ERα and DES-ERα LBD complexes (outside of helix 12 and the loop connecting helices 11 and 12), the GEN-

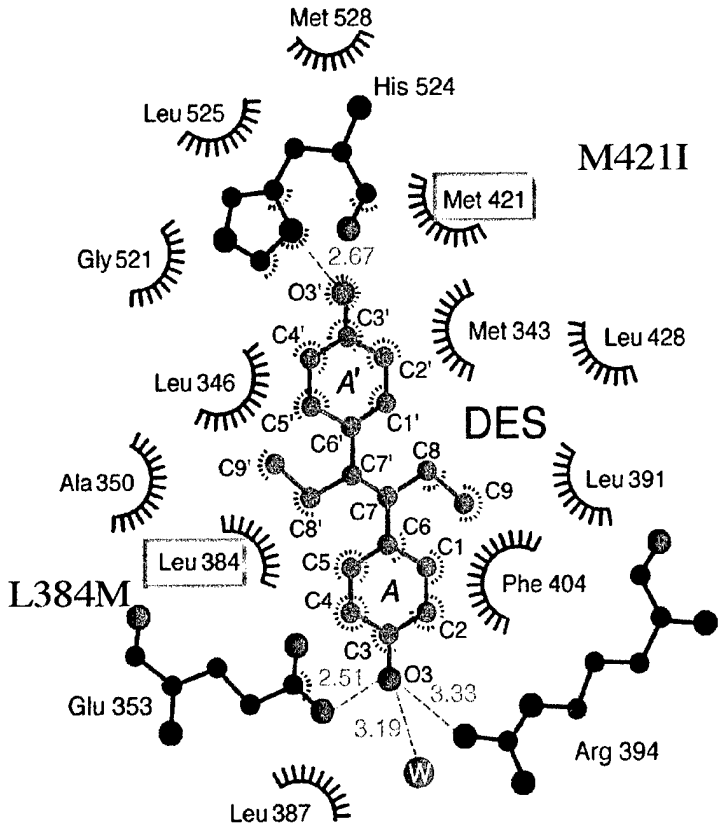

Figure 3. Comparison of amino acids that line the ligand-binding pocket of ERα LBD and ERβ LBD.

ERβ LBD complex (2) may serve as the basis for a model of the agonist-bound conformation of the ERβ LBD. In order for the A' ring region of R,R-THC to fit between the side chains of Met 336 and Ile 373 (in this model), R,R-THC would need to be positioned lower in the ERβ binding pocket relative to its location in the ERα binding pocket. This is because the side chain of Met 336 is larger than that of Leu 384, and conversely the side chain of Ile 373 is smaller than that of Met 421. This lower position should be highly unfavorable because it would result in a steric clash between both atoms of one of the R,R-THC ethyl groups with the side chain of Leu 298 at the floor of the pocket (**Appendix, Figure 5**). To avoid this unfavorable interaction, R,R-THC binds in the alternative mode observed in the crystal. Consistent with this hypothesis, the dimethyl analog of R,R-THC, which should be less sterically hindered from adopting the lower position, acts as a weak ERβ partial agonist (7).

Because the conformational effects of ligand binding are cooperative in nature, residues that lie outside the binding pocket may also play roles the subtype-selective effects of R,R-THC. Nevertheless, preliminary mutagenesis results indicate that Leu 384/Met 336 and Met 421/Ile 373 do contribute to these effects (**Figure 4**), at least for ERα. However, the effect is only partial for ERα and the corresponding ERβ → ERα amino acid swapping mutations (Met336 → Leu384, Ile373 → Met421) do not result in the recovery of any detectable R,R-THC agonist activity (**Figure 4B**). The same result was observed both for full length ERα and ERβ (data not shown) as well as for the LBD of each ER fused to the Gal4 DNA binding domain. In both cases, a transcriptional activation assay was used to assess ER or ER LBD activity in transiently transfected MDA-MB-231 breast cancer cells in which recombinant GRIP1 was also c0-expressed (see attached manuscript for experimental details). Estradiol and OHT were tested as positive and negative controls, respectively for both ER subtypes and behaved as expected with both single and double mutations (**Figure 4A**). In total, these results indicate that the two non-conserved amino acids that line the inside of the LBD pocket (**Figure 3**) are not sufficient for selective α or β behavior in terms of response to R,R-THC. However, for other ligands, such as HPTE (**Figure 5**), these two amino acids do appear to play a major role in distinguishing between ERα and ERβ behavior (**Figure 4B**). While HPTE, an estrogenic metabolite of the

organochlorine pesticide methoxychlor, is a potent ERα agonist in HepG2 cells (15), it has minimal agonist activity with either human or rat ERβ and almost completely abolishes estradiol-induced ERβ activity. Notably, exchange of the two ERα/β-distinguishing amino acids effectively converted ERα into ERβ and vice versa in a cell based reporter assay of HPTE activity (**Figure 4B, HPTE**). In contrast, the behavior of certain pyrazole derivatives, like

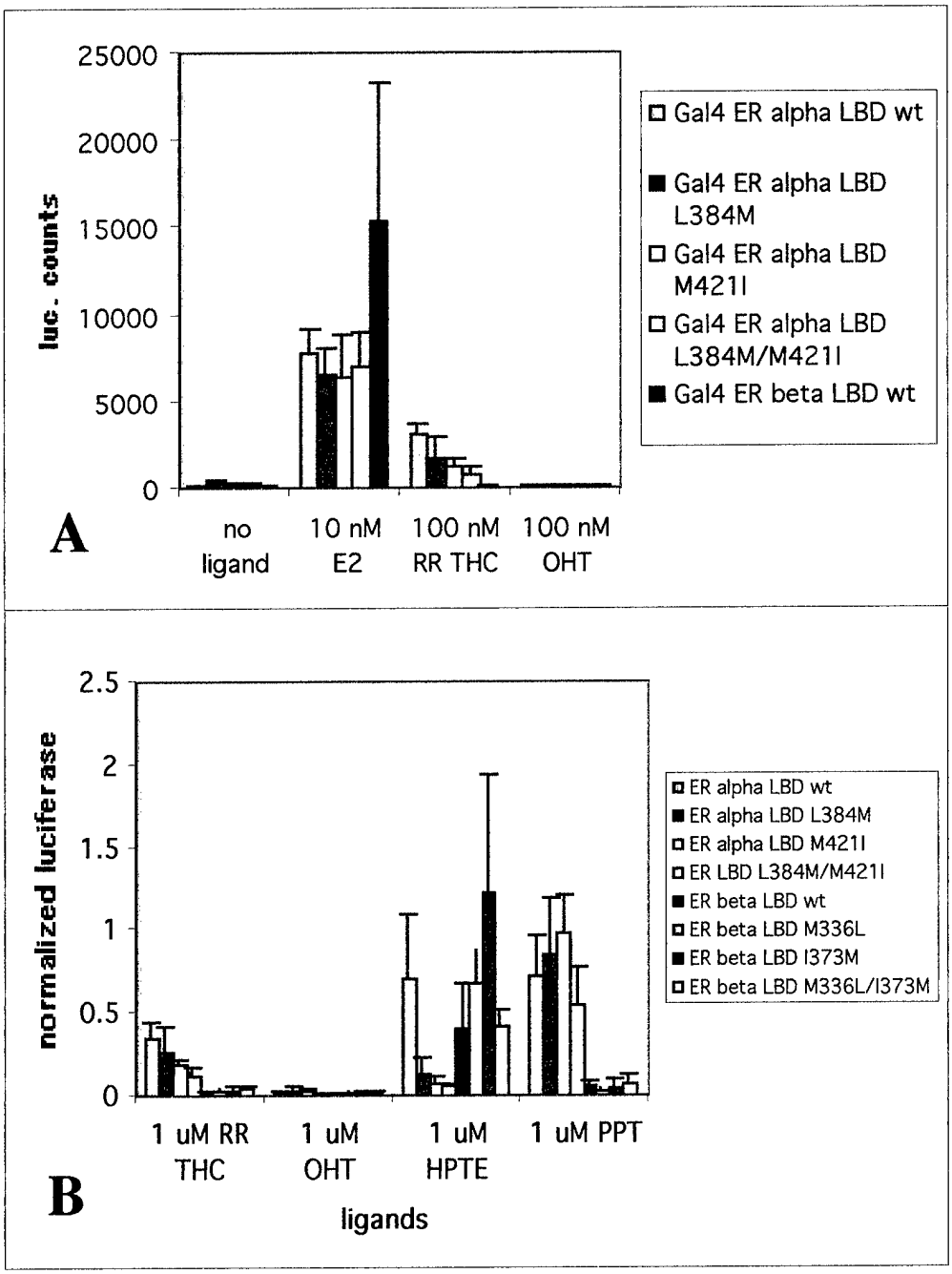

Figure 4. Transcriptional activity of Gal4-ERα/β LBD mutants versus wild type ERα/β LBDs (+ GRIP1; in MDA-MB231 cells). **A.** Positive and negative controls. **B.** ER subtype-selective ligands.

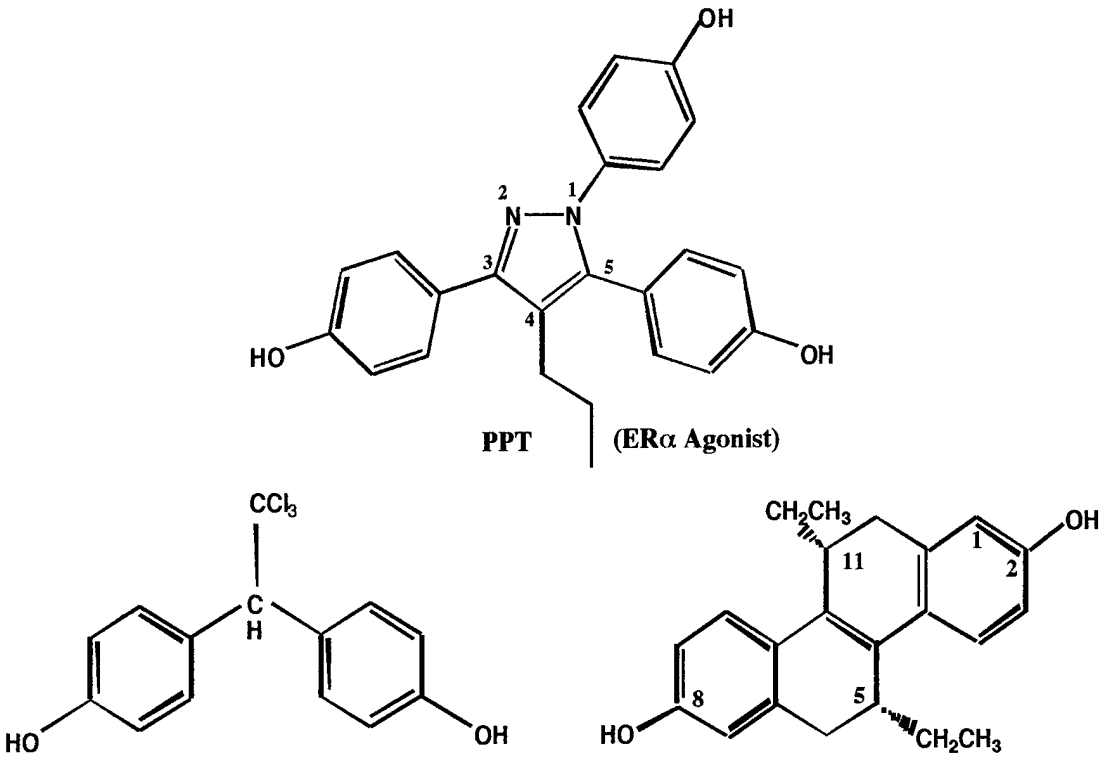

Figure 5. Structures of ERα/β subtype-selective agonists and antagonists. HPTE = *p*-hydroxyphenyl-trichloroethane. PPT = propylpyrazole triol.

propyl pyrazole triol (**Figures 4B and 5, PPT**), which have been shown to be selective ERα agonists with little or no affinity for ERβ (16), are unaffected by exchange of these two amino acids. The structural basis for these differences has yet to be determined.

Perhaps these contrasting outcomes are not quite as surprising they appear since we and others have data to show that the two ER subtypes seem to prefer different default conformations (**Appendix, Figure 2B**) and that even for the same ER subtype, structurally similar ligands (e.g. OHT and RAL) can induce unique and subtly different conformations. Therefore, it is very likely that amino acids outside the LBD pocket contribute to ligand discrimination and ER subtype-selective behavior. Additional proof that these subtle conformational differences are real and significant comes from the development of peptides (generated by phage display) that can distinguish between complexes of ERα or ERβ bound to these ligands (17, 18). For example, a peptide designated α/β V can distinguish between the OHT-ERα/β complexes and other SERM-ER complexes, including Ral-ERα/β (17). Other peptides (e.g. 7β-16) can selectively recognize GW5638-ERα/β compared to other SERM-ER complexes (18). One of our major goals is to understand the structural/molecular basis for these differences. Thus, one approach is to determine the crystallographic structures of ERα/β LBD complexes with these ligand-selective peptides. In addition, we will continue to exploit site-directed mutagenesis of both ER subtypes to better define which residues distinguish ERα from ERβ in response to ligands that have unique activity- and/or ER subtype-selective behavior. This type of

information will be essential for our compound modeling and drug design efforts. We will want to know, for example, the minimum number of amino acids required for ER subtype selection for each of the compounds mentioned above. We will also need to know more about the conformational flexibility of the two ER subtypes and how that flexibility distinguishes between coactivator and corepressor preference.

KEY RESEARCH ACCOMPLISHMENTS

- Extensive refinement of crystallographic structures of ERα and ERβ LBDs bound to R,R-THC, an ERα agonist/ERβ antagonist. New insights into mechanisms of agonism and antagonism revealed.
- Molecular basis of novel concept "passive antagonism", or antagonism without side chains, predicted from structures of ERα and ERβ LBDs bound to R,R-THC and TIF2/GRIP1 NR box II peptide.
- Novel concept of helix 12 dynamic equilibrium: Positioning of helix 12 is more variable than previously believed and is a key discriminator among diverse agonist- and antagonist-induced conformations of ERα and ERβ. Hydrophobic cleft in the LBD can accommodate coactivators (agonists), corepressors (antagonists), and/or different orientations of helix 12 in the presence of different antagonists.
- Transcriptionally active conformation of ER LBD requires cooperative participation of ligand, LBD, and coactivator.
- Molecular basis of ER subtype-selective agonism and antagonism predicted from structure of ERα and ERβ LBDs bound to R,R-THC. R,R- THC binds to the ERβ LBD without nucleating many of the equivalent cooperative interactions observed in the ERα complex that reflect agonist behavior.
- Preliminary mutagenic analysis of ERα and ERβ LBDs reveals that ligand-selective α and β character, especially transcriptional activity, depend on more than the two amino acids that differ in the ligand binding pockets of the two ERs.

REPORTABLE OUTCOMES

- Manuscripts, Abstracts, Presentations:
  A manuscript detailing the results reported here was recently submitted to *Nature Structural Biology* for review. It is included in the Appendix.
  Abstracts for two meetings are included in the Appendix.
  Presentations (no abstract): Invited speaker at various academic institutions and meetings.
  Symposium Speaker: Mini-Symposium II, 23rd Annual San Antonio Breast Cancer Symposium, San Antonio, TX, 12/00.
- Patents: none
- Degrees obtained with award support: none
- Development of cell lines, etc: none
- Informatics:
  R,R-THC-ERα/β crystallographic coordinates will be deposited in the PDB and will be released upon publication of the structures.
- Funding applied for based on work supported by this award:
  An R01 proposal (CA89489), "Development and Characterization of Novel SERMs" has been approved for funding by the NCI and funding should begin in August. This work

will complement and extend, but not duplicate, the structural work represented by this DOD award.

- Employment or research opportunities applied for: none

CONCLUSIONS

The major goal of this investigation is to determine the three-dimensional structures of the two known human estrogen receptors (ERα and ERβ) complexed with receptor-selective estrogens and antiestrogens (SERMs). The crystallographic structures of ERα and ERβ ligand binding domains complexed with *cis*-R,R-diethyl-tetrahydrochrysene-2,8-diol (R,R-THC) have been solved and the data extensively refined, suggesting novel mechanisms by which this compound can act as an ERα agonist and as an ERβ antagonist. Agonists and antagonists bind at the same site within the core of the ER LBD to induce distinct conformations in the transactivation domain (AF-2), especially in the positioning of helix 12. Previously determined structures of ERα with 4-hydroxytamoxifen (OHT) and diethylstilbestrol (DES) revealed and defined a multipurpose docking site on ERα and ERβ that can accommodate either helix 12, in the presence of OHT, or one of several co-regulators in the presence of DES. R,R-THC stabilizes a conformation of the ERα LBD that favors coactivator association and a conformation of the ERβ LBD that prevents coactivator association. A comparison of the two structures, combined with functional data, reveals that R,R-THC does not act on ERβ through the same mechanisms used by other known ER antagonists. Instead, R,R-THC antagonizes ERβ through a novel mechanism we term "passive antagonism". Importantly, R,R- THC binds to the ERβ LBD without nucleating many of the equivalent cooperative interactions observed in the ERα complex that reflect agonist behavior. Paradoxically, the R,R-THC-ERβ structure is very similar to the structure induced by genistein, which acts as a partial estrogen through both ER subtypes. Clearly, formation of the transcriptionally active conformation of ER LBD requires cooperative participation of ligand, LBD, and coactivator. Ongoing mutagenesis studies should help define the molecular and structural differences that are responsible for these unanticipated results. The passive antagonism mechanism suggests a novel approach to the design of ligands that selectively antagonize the two ER subtypes. Such ligands may have novel therapeutic properties that can be exploited to prevent or treat breast cancer.

So what? It is clear from the various solved structures for ERα and ERβ bound to diverse SERMs that the resulting ER conformations and ligand pharmacologies are not as simple as previously believed. Rather than just two distinct conformations of ER that reflect agonism or antagonism, it appears that multiple conformations are possible, each of which may mediate a different pharmacology for a particular ligand in a given tissue, such as the breast, uterus, bone, or cardiovascular system. It has become clear that variable antagonism can be achieved both by the presence of bulky side chains and by molecules that lack bulky side chains. Thus, it is important to solve additional structures for ERα and ERβ complexed with ligands that have been shown to have different tissue-selective pharmacologies, such as GW5638 and ICI 182,780 (Faslodex). In addition, structure-based mutagenic analyses of the two ER subtypes will help define residues that contribute to α and β activity-selective and subtype-selective behavior. Such information is essential to design compounds that have defined properties. A major goal of this investigation is to characterize and/or design compounds that are useful as chemopreventive agents, especially for breast and uterine cancers. To be useful in a clinical setting, such as

hormone replacement therapy, such compounds must also have the beneficial effects of estrogen, including maintenance of bone density, protection of the cardiovasculature, prevention of hot flashes and perhaps delayed onset of Alzheimer's disease and maintenance of cognitive function. It seems unlikely that a single molecule will have all of these properties. However, it is clear that improved SERMs can be designed and/or discovered. Since most, if not all, of the behavior of the two known ERs is dictated by the conformations induced by diverse natural and synthetic ligands, determination and analysis of the corresponding 3D structures for representative SERMs should be a valuable tool for dissecting underlying molecular mechanisms and for designing new generation compounds. We will therefore continue our efforts to determine and correlate ER-SERM structures with their known biological activities and to understand the unique structural/molecular characteristics that define and differentiate ERα and ERβ.

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APPENDIX

Manuscript submitted to *Nature Structural Biology*:

Andrew K. Shiau, Danielle Barstad, James T. Radek, Marvin J. Meyers, Benita S. Katzenellenbogen, John A. Katzenellenbogen, David A. Agard, and Geoffrey L. Greene. Structural Characterization of an Estrogen Receptor α Agonist/Estrogen Receptor β Antagonist Reveals a Novel Mode of Receptor Antagonism.

Selected abstracts of presentations at national or international meetings:

1. 14th International Symposium of the Journal of Steroid Biochemistry & Molecular Biology, Quebec City, Canada, 6/00.

2. 14th Annual Symposium on the Long-Term Effects of Estrogen Deprivation, Telluride, Colorado, 7/00.

3. Hormones and Cancer 2000, Port Douglas, Australia, 11/00.

4. LAMPOSIUM 2001, Cincinnati, Ohio, 3/01.

5. EMBO Workshop on Nuclear Receptor Structure and Function, Erice, Sicily, Italy, 5/01.

Structural Characterization of an Estrogen Receptor α Agonist/Estrogen Receptor β Antagonist Reveals a Novel Mode of Receptor Antagonism

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Nuclear receptor (NR) ligands regulate the transcriptional activity of NRs by stabilizing distinct conformations of the ligand binding domains (LBDs) of their cognate receptors. NR agonists favor LBD conformations that allow NRs to interact with transcriptional coactivator proteins, essential mediators of the transcriptional activation process. By contrast, NR antagonists favor LBD conformations that inhibit the interaction of NRs with coactivators. The R,R enantiomer of 5,11-*cis*-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (THC) was recently identified as an estrogen receptor (ER) ligand that differentially affects the transcriptional activity of the two ER subtypes; THC functions as an ERα agonist and as an ERβ antagonist. We have determined the crystal structure of the ERα LBD bound to both THC and a peptide containing the NR box II region of the coactivator GRIP1 and that of the ERβ LBD bound to THC. THC stabilizes a conformation of the ERα LBD that favors coactivator association and a conformation of the ERβ LBD that prevents coactivator association. A comparison of the two structures, combined with functional data, reveals that THC does not act on ERβ through the same mechanisms used by other known ER antagonists. Instead, THC antagonizes ERβ through a novel mechanism we term "passive antagonism".

Estrogens play central roles in the differentiation, development, and homeostasis of a wide variety of vertebrate tissues[1]. Currently, a number of estrogenic compounds are being used to regulate human fertility and to treat human diseases, including osteoporosis, cardiovascular disease, and breast cancer **(ref)**. The physiological effects of both endogenous and synthetic estrogens are mediated by the estrogen receptors (ERs), ERα and ERβ, members of the nuclear receptor (NR) superfamily of ligand-regulated transcription factors. The two ERs have overlapping but distinct tissues distribution patterns *in vivo* and distinct activation profiles at both classical and complex promoter elements **(ref)**. Hence, it is unclear how ERα and ERβ individually contribute to the effects of known estrogens. Ligands that act differentially on ERα and ERβ would be potentially

valuable both as tools to dissect the biological roles of the two ERs and as novel therapeutics with pharmacological properties distinct from existing drugs.

In a search for ER subtype-selective ligands, the R,R enantiomer of 5,11-*cis*-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (THC) (Figure 1A) was identified as a compound with a significant affinity preference for ERβ over ERα (K_d = 1.3 nM and 0.2 nM for ERα and ERβ, respectively), and profoundly different activation profiles on the two ERs[2,3]. THC functions as an agonist on ERα with an EC_{50} ~ 3 nM but has no effect on the transcriptional activity of ERβ. Instead, this compound potently antagonizes the effects of the endogenous estrogen, 17β-estradiol (E2), on ERβ with an IC_{50} < 10 nM[2,3]. Consistent with these subtype-specific effects on transcriptional activation, THC also differentially affects the two ERs in protease protection and coactivator recruitment assays[4]. However, the molecular basis of the subtype-dependent behavior of the compound is still not understood. Here we describe structural and further functional characterization of the effects of THC on ERα and ERβ. These studies indicate that THC exerts its effects on the transcriptional activity of the two ERs by differentially affecting the structures of the two receptors and that THC antagonizes ERβ through a mechanism distinct from those used by other known ER antagonists.

Subtype-selective Transcriptional Effects of THC

The transcriptional activity of ERα results from at least two functionally separable transcriptional activation functions (AFs), AF-1 within the N terminus and AF-2 within the C-terminal ligand binding domain (LBD)[5,6]. By contrast, the transcriptional activity of ERβ is largely dominated by that of AF-2 within its LBD[7,8]. AF-1 activity is regulated by growth factor-inducible phosphorylation by MAP kinases[9], while AF-2 activity is regulated by the binding of ligands **(ref)**. Pure agonists, such as E2 and the synthetic nonsteroidal estrogen diethylstilbestrol (DES), trigger AF-2 activity while pure antagonists, such as ICI 164,384 (ICI), and mixed-

function partial agonist/antagonists, such as 4-hydroxytamoxifen (OHT) and raloxifene (RAL), block AF-2 activity (Figure 1A) **(ref)**. AF-1 and AF-2 may contribute independently, additively, or synergistically to the activity of ERα depending on the ligand and promoter context **(ref)**. As a result, the agonist activity of THC may arise from the stimulation of AF-1 and/or AF-2.

To determine if THC regulates ERα AF-2 activity, a series of transcriptional assays were performed using fusions between the DNA binding domain (DBD) of the yeast transcriptional activator, GAL4, and the ERα LBD. For comparison, equivalent assays were performed using a similar fusion between the GAL4 DBD and the ERβ LBD. E2 stimulates the transcriptional activity of both the ERα and ERβ LBD chimeras (Figure 1B). By contrast, THC stimulates the transcriptional activity of the ERα LBD fusion (albeit to a lesser degree than E2) but has no effect on the transcriptional activity of the ERβ LBD chimera (Figure 1B). Consistent with its effects on full-length ERβ[2,3], THC also antagonizes the effects of E2 on the ERβ LBD chimera (data not shown). Thus, THC affects the AF-2-derived transcriptional activities of the two ERs in a subtype-selective manner.

Overall Structures of the THC-ER LBD Complexes

Recent structural studies indicate that NR ligands regulate AF-2 activity by modulating the structure of NR LBDs. NR ligands bind to their cognate receptors within a hydrophobic pocket formed within the core of the narrower or "lower" half of the LBD. The binding of an AF-2 agonist to an NR LBD triggers the repositioning of the most C-terminal helix of the LBD, helix 12, such that it lies across the entrance to the ligand binding pocket, sealing in the agonist[10]. This conformation of the LBD allows the receptor to interact with a class of proteins known as transcriptional coactivators, which enhance ligand-dependent transcription of NRs[11]. Members of the p160 family of coactivators[11], including SRC-1/NCoA1, GRIP1/TIF2/NCoA2, and p/CIP/RAC3/ACTR/AIB1, recognize agonist-bound NR LBDs via short sequence motifs, LXXLL (where L is leucine and X is any amino acid) called NR

boxes[12-15]. These NR boxes form amphipathic α-helices which recognize a hydrophobic groove on the surface of an agonist-bound LBD formed by residues from helices 3, 4, 5, and 12[16-19].

By contrast, NR AF-2 antagonists stabilize alternative LBD conformations that sterically preclude coactivator recognition. The structures of the BMS614-retinoic acid receptor α[20], OHT-ERα[19], RAL-ERα[21], RAL-ERβ[22], and ICI-ERβ LBD[23] complexes reveal that certain portions of these compounds, termed extensions or side chains, cannot be contained within the ligand binding pocket. Instead, these side chains project out of the entrance to the binding pocket, which is covered by helix 12 in the agonist-bound LBD structures. Hence, helix 12 is precluded from adopting the "agonist-bound" conformation. In addition, the structures of the BMS614, OHT, and RAL complexes show that these antagonists also induce structural distortions in and around the binding pocket, that favor an extended conformation of the segment of peptide backbone which forms the C-terminal end of helix 11 in the agonist-bound structures. This "unraveling" of helix 11 allows helix 12 to reach the static region of the coactivator recognition groove formed by helices 3, 4, and 5 and occlude the recognition groove by mimicking the interactions of the NR box with the LBD[19-22]. ICI affects helix 12 positioning in a different manner. In its complex with the ERβ LBD, the terminal n-butyl moiety of the ICI side chain embeds itself in the recognition groove, preventing both coactivator binding and the helix 12 conformation observed in the other antagonist complexes. As a result, helix 12 is completely disordered in this complex[23].

Curiously, the recent structure of the ERβ LBD bound to the ERβ partial agonist genistein (GEN) reveals that ligand binding may support yet a third conformation of helix 12[22]. Helix 12 is bound over the ligand binding pocket in a position that only partially occludes the coactivator recognition surface. The functional significance of this conformation of the LBD is unclear.

These structural data and the behavior of THC in transactivation assays suggest a striking prediction: the same ligand, THC, should be able to both stabilize a

conformation of the ERα LBD that favors coactivator binding and a conformation of the ERβ LBD that precludes coactivator binding. Notably, THC, unlike all other known ER antagonists, such as OHT, RAL, and ICI, lacks a bulky extension or side chain (Figure 1A), so THC must act on ERβ by fundamentally different mechanisms than these other antagonists.

In order to verify this prediction, crystals of the ERα LBD bound to both THC and a peptide derived from the second NR Box of the coactivator GRIP1 and of the ERβ LBD bound to THC were grown and both structures determined by molecular replacement. The ERα complex structure has been refined to a crystallographic R factor of 20.3% (R_{free} = 24.2%) using data to 1.95 Å and the ERβ complex structure has been refined to a crystallographic R factor of 26.5% (R_{free} = 30.4%) using data to 2.85 Å (Table 1 and Figure 4A).

When bound to THC, the ERα LBD adopts the same conformation as it does when it is bound to the full agonists E2 and DES (Figure 2A)[19,21]. The LBD is a wedge-shaped molecule composed of a small beta hairpin and eleven to twelve helices arranged in three layers. Helix 12 adopts the agonist-bound conformation in the THC-ERα LBD complex; helix 12 packs against helices 3, 5, 6, and 11, covering the entrance to the ligand binding cavity. THC is completely enveloped by the binding cavity within the lower subdomain of the LBD formed by residues from helices 3, 6, 7, 8, 11, and 12 and the beta hairpin (Figure 2A). As in the DES-ERα LBD-GRIP1 peptide complex[19], the coactivator peptide is bound in an α-helical conformation to a hydrophobic groove on the surface of the LBD formed by residues from helices 3, 4, 5, and 12 (Figure 2A).

As described previously, the ERβ LBD shares the same overall fold as the ERα LBD (Figure 2A)[22]. And like the ERα LBD, the ERβ LBD recognizes THC with a ligand binding pocket located within its lower subdomain. However, the binding of THC to the ERβ LBD does not stabilize the agonist-bound conformation of helix 12

(Figure 2A). Neither is helix 12 bound to the portion of the coactivator recognition groove formed by helices 3, 4, and 5 as it is in the OHT-ERα LBD[19], RAL-ERα LBD[21], and RAL-ERβ LBD[22] complexes (Figure 2B) , nor is it disordered as it is in the ICI-ERβ LBD complex[23]. Instead, helix 12 in the THC-ERβ LBD complex interacts with the rest of the LBD in a manner most like that observed in the GEN-ERβ LBD complex (Figure 2B)[22].

Helix 12 is positioned similarly, and of comparable length (residues 487 to 498 in the THC complex and residues 487 to 497 in the GEN complex[22]) in the two structures (Figure 2B). At the N-terminal end of helix 12, the side chain of Val 487 (which packs against the side chains of Leu 306 and Trp 335 in the THC complex) is inserted into the entrance to the ligand binding pocket formed by helices 3, 5/6, and 11 in both complexes. This causes the C-terminal end of helix 12 to project away from the rest of the LBD. As a result, the side chains of Leu 491, Met 494, and Leu 495, which mimic the interactions of the three NR box leucines with the static region of the coactivator recognition groove in the RAL-ERβ LBD complex[22], only partially occlude the coactivator binding site in the GEN[22] and THC complexes. In the THC complex, the side chain of Leu 491 packs against that of Trp 335. The side chain of Met 494 packs against those of residues on helix 3 (Asp 303, Leu 306, and Val 307) and the side chain of Leu 495 is embedded within the coactivator binding groove and packs against the side chains of Ile 310, Val 328, Leu 331, and Glu 332.

Ligands and Helix 12 Positioning

The conformation of the ERβ LBD observed in both the THC and GEN complexes should be incapable of interacting with coactivators and, hence, transcriptionally silent for two reasons. The residues from helix 12 (Leu 490, Glu 493, and Met 494) that are predicted to form part of the coactivator recognition surface are inappropriately positioned, and helix 12 itself is bound such that it partially occludes the static region of the coactivator binding surface formed by residues from helices 3, 4, and 5. Yet, GEN and THC clearly exhibit different activities on ERβ in

mammalian cells; GEN acts as a partial agonist[8] and THC acts as a pure antagonist[2,3]. How can the similarity of the two structures be reconciled with the different activities of these compounds in transcriptional assays?

The simplest model that explains these and other data is based on two suppositions (Figure 3A). First, helix 12 in the unliganded ERβ LBD is in equilibrium between the THC/GEN-bound conformation, which precludes coactivator association, and the agonist-bound conformation seen in the E2- and DES-ERα LBD structures, which favors coactivator association (with the "inactive" THC/GEN-bound conformation being heavily favored). Second, rather than stabilizing a single static conformation of helix 12, ligands serve to shift the balance of this equilibrium.

According to this model, helix 12 in the unliganded or apo-ERβ LBD, although largely in a transcriptionally silent conformation, may also adopt a transcriptionally active one (Figure 3A). Hence, the low but detectable constitutive AF-2-derived transcriptional activity of the apo-LBD would result from the modest but significant affinity of the apo-LBD for coactivator[8]. Full agonists, such as E2 and DES, shift the helix 12 conformational equilibrium heavily in favor of the "active" conformation, and stimulate AF-2 activity by increasing the affinity of the LBD for coactivator (Figure 3A). Because they are incapable of shifting the equilibrium in favor of the active conformation to the same extent as full agonists, partial agonists, such as GEN, are only able to incrementally increase the affinity of the receptor for coactivator and, hence, activate AF-2 less efficiently than full agonists (Figure 3A). GEN binding should allow the ERβ LBD to sample both inactive and active conformations of helix 12 (possibly to comparable extents). The fact that the inactive THC/GEN-bound conformation is observed in the crystal structure may reflect the true bias of the equilibrium in solution or it may be the result of the influence of the crystallization conditions on the equilibrium.

In principle, THC could antagonize receptor activity by shifting equilibrium in favor of the inactive THC/GEN-bound conformation (Figure 3A). If THC binding

forces the receptor to favor this conformation even more than it does in the absence of ligand, the THC-receptor complex would be expected to have an even lower affinity for coactivator than the unliganded receptor. This would be consistent with the absence of transcriptional activity of the THC-ERβ complex and the conformation of helix 12 observed in the crystal.

The helix 12 conformational equilibrium would be difficult to observe directly. Nonetheless, the mechanisms of the various ligands, inferred from the model described above, suggest a testable hypothesis: the binding of different ligands should modulate the affinity of the ERβ LBD for coactivator such that E2-LBD ~ DES-LBD > GEN-LBD > apo-LBD > THC-LBD. The affinities of various ligand-ERβ LBD complexes for an LXXLL motif-containing peptide were directly measured using a fluorescence polarization-based binding assay (Figure 3B)[24]. The full agonist complexes bind the peptide approximately 1.5-fold more tightly than the GEN complex (K_d[E2] = 0.36 μM and K_d[DES] = 0.41 μM versus K_d[GEN] = 0.58 μM). In addition, the GEN complex binds the peptide more tightly than the unliganded receptor (K_d[apo] = 2.1 μM), whereas the THC complex (K_d[THC] = 3.7 μM) binds the peptide significantly weaker than the unliganded receptor. Thus, these and other previously described biochemical data are entirely consistent with the described helix 12 conformational equilibrium model. Furthermore, recent mutational and crystallographic data suggest that the positioning of helix 12 in ERα[25] and other NRs[10] is also dictated by a ligand-sensitive conformational equilibrium.

THC Interaction with the ERα and ERβ LBDs

THC binding stabilizes a transcriptionally active conformation of the ERα LBD and a transcriptionally inactive one of the ERβ LBD. The conformation of an NR LBD stabilized by a particular ligand is the direct structural consequence of the interactions formed between that ligand and the binding pocket of that protein.

Consistent with these ideas, THC interacts with the ligand binding pockets of the two ERs in substantially different ways.

The ERα LBD recognizes THC much as it does the agonists, E2[21,25,26] and DES[19] (Figures 4B and 4C). The A ring of THC is bound in approximately the same position as the A rings of E2 and DES near helices 3 and 6. The A ring itself interacts with the side chains of Ala 350, Leu 387, Leu 391, and Phe 404 and the A ring phenolic hydroxyl forms hydrogen bonds to the side chain carboxylate of Glu 353, to the guanidinium group of Arg 394, and to a buried water molecule. The A′ ring of THC is bound by the opposite end of the binding pocket near helices 7, 8, and 11 which binds the D ring of E2 and the A′ ring of DES. The A′ ring forms van der Waals contacts with Met 421, Ile 424, Gly 521, His 524, and Leu 525, and the phenolic hydroxyl hydrogen bonds with the side chain imidazole of His 524.

Although THC, E2, and DES form many of the same interactions with ERα, the three ligands differ in their ability to fill the binding pocket. In the E2 complex, there are unoccupied cavities adjacent to the α face of the B ring and the β face of the C ring of E2[21,25,26]. DES is able to fill both of these cavities with its two ethyl groups[19]. One of the ethyl groups of THC fills the B ring α face cavity by forming packing interactions with Leu 391, Phe 404, Met 421, Phe 425, and Leu 428 (Figures 4B and 4C). However, due to the *cis* stereochemistry of the molecule, the other ethyl group of THC points towards the α face, instead of the β face, of the C ring region of the binding pocket. Therefore, this ethyl group is unable to fill the cavity near the C ring, and instead packs against the side chains of Leu 346 and Met 421 (Figures 4B and 4C). In order to accommodate these additional interactions, the THC scaffold is tilted approximately 10° away from the floor of the binding pocket (and Leu 346) relative to the plane of the E2 scaffold. This suboptimal alignment may result in the reduced agonist activity of THC (relative to E2) on ERα. This idea is supported by the observation that the *trans* analog of THC, whose ethyl groups should be positioned like those of DES, acts as a full agonist on ERα[2].

THC binds to the two ERs in a grossly similar manner (Figure 4C). However, the two ethyl groups of THC adopts significantly different conformations in the two complexes. Whereas the two ethyl groups are rotated underneath the tetrahydrochrysene scaffold in the ERα complex, they are rotated outwards away from the body of THC in the ERβ complex (Figures 4A and 4C). As it does in the ERα complex, one ethyl group fills the α face B ring cavity in the ERβ complex and forms nonpolar contacts with Leu 343, Phe 356, and Leu 380 (Figures 4B and 4C). But, in order to accommodate its extended conformation, the tetrahydrochrysene scaffold is displaced both towards the entrance of the binding pocket (near helices 3, 6, and 11) and away from the floor of the binding pocket relative to its position in the ERα complex (Figure 4C).

This displacement significantly affects the interaction of THC with the ERβ ligand binding pocket in several ways. First, it allows the other ethyl group not only to pack against Leu 298 (equivalent to Leu 346 in ERα), but also to Thr 299 at the entrance to the binding pocket (Figure 4B). Even more importantly, because of its altered positioning, the tetrahydrochrysene scaffold of THC is only able to interact with a subset of the equivalent ERβ LBD residues that it interacts with in the ERα complex (Figures 4B and 4C). The A ring of THC is bound in the region of the ERβ binding pocket near helices 3 and 6, as it is in the ERα complex. But in the ERβ complex the THC A ring only interacts with three (Ala 302, Leu 339, and Phe 356, equivalent to Ala 350, Leu 387, and Phe 404, respectively) of the four residues that it packs against in the ERα complex. In the ERα complex, the phenolic A ring hydroxyl forms hydrogen bonds with the side chains of Glu 353, Arg 394, and a buried water molecule. Because it is bound somewhat more distantly from the equivalent residues (Glu 305 and Arg 346) in the ERβ complex, the A ring hydroxyl is unable to interact with the equivalent buried water and only forms hydrogen bonds to the side chains of Glu 305 and Arg 346. At the opposite end the the pocket, the A′ ring of THC in the ERβ complex packs against three (Gly 472, His 475, and Leu

476, equivalent to Gly 521, His 524, and Leu 525, respectively) of the five residues it interacts with in the ERα complex. Because it is displaced by approximately 1 Å relative its location in the ERα complex, the A′ ring phenolic hydroxyl is unable to hydrogen bond with the imidazole of His 475. Instead, the A′ ring hydroxyl forms a weak hydrogen bond to the carbonyl of Gly 472.

When they bind to ERα (and presumably ERβ), ER agonists favor the agonist-bound conformation of helix 12, but they do so indirectly[19,21]. An ER agonist forms hydrogen bonds and van der Waals contacts with residues from helices 3, 5, 6, 11 and the beta turn, stabilizing these residues in conformations that allow them to interact with the ligand, with each other, and with other residues in and around the binding pocket. The formation of this series of cooperative interactions orients residues from helices 3, 5, 6, and 11 such that they create a predominantly hydrophobic binding surface for helix 12. This results in a shift in the helix 12 conformational equilibrium towards the agonist-bound conformation. From a structural standpoint, THC acts as an ERα agonist. When bound to ERα, THC fulfills all of the hydrogen bonding and nonpolar interactions observed in the E2 and DES complexes that nucleate the multiple cooperative interactions in and around the binding pocket that favor the agonist-bound conformation of helix 12.

By these criteria, THC does not behave as an ERβ agonist. As described above, THC binds to ERβ without interacting with several residues that it contacts in ERα, and by forming alternative hydrogen bonds and additional packing contacts not observed in the ERα complex (Figures 4B and 4C). Hence, THC is able to bind to the ERβ LBD without nucleating many of the equivalent cooperative interactions observed in the ERα complex. For example, in the ERα complex the side chain of Met 528 packs against the side chains of His 524 and Met 343, stabilizing the helical conformation of the flanking residues. Because THC binding favors alternative conformations of His 475 and Met 295 (equivalent to His 524 and Met 343, respectively), the side chain of Met 479 (equivalent to Met 528) is completely

disordered in the ERβ complex. In fact, by stabilizing conformations of binding pocket residues that prevent their interaction, THC binding should actually disfavor the equilibrium to the agonist-bound conformation of helix 12 (Figures 3A and 3B).

What differences between the two ERs allow THC to stabilize these distinct arrangements of ligand binding pocket residues? There are only two binding pocket residues which are not identical in the two subtypes: Leu 384 (from helix 5/6) and Met 421 (from helix 8) in ERα, which are equivalent to Met 336 and Ile 373, respectively, in ERβ[27-29]. These two residue pairs are similarly positioned on opposite faces of THC in their respective complexes. In the ERα complex, Leu 384 and Met 421 form several nonpolar contacts with THC (Figures 4B and 4C). By contrast, only Met 336 interacts with THC in the ERβ complex (Figures 4B and 4C).

Modeling suggests that Met 336 and Ile 373 would adopt both different positions and different conformations (relative to their positions in the THC-ERβ complex) in the agonist-bound conformation of the ERβ LBD (Figure 5). There is currently no known structure of ERβ bound to a full agonist. However, given its similarity to the E2-ERα[21] and DES-ERα LBD complexes[19] (outside of helix 12 and the loop connecting helices 11 and 12), the GEN-ERβ LBD complex[22] may serve as the basis for a model of the agonist-bound conformation of the ERβ LBD. In order for the A' ring region of THC to fit between the side chains of Met 336 and Ile 373 (in this model), THC would need to be positioned lower in the ERβ binding pocket relative to its location in the ERα binding pocket. This is because the side chain of Met 336 is larger than that of Leu 384, and conversely the side chain of Ile 373 is smaller than that of Met 421. This lower position should be highly unfavorable because it would result in a steric clash between both atoms of one of the THC ethyl groups with the side chain of Leu 298 at the floor of the pocket (Figure 5). To apparently avoid this unfavorable interaction, THC binds in the alternative mode observed in the crystal. Consistent with this hypothesis, the dimethyl analog of

THC, which should be less sterically hindered from adopting the lower position, acts as a weak ERβ partial agonist[2].

Because the conformational effects of ligand binding are cooperative in nature, residues that lie outside the binding pocket may also play roles the subtype-selective effects of THC. Nevertheless, preliminary mutagenesis results indicate that Leu 384/Met 336 and Met 421/Ile 373 do contribute to these effects (unpublished results).

"Passive" Antagonism - Antagonism Without Side Chains

The positioning of the side chains of OHT, RAL, and ICI directly or "actively" preclude the agonist-bound conformation of helix 12 by steric hindrance. Hence, we define their common mechanism of action as "active antagonism". Clearly, THC does not have a side chain (Figure 1A), and in its complex with ERβ, helix 12 is not sterically precluded from adopting the agonist-bound conformation as it is in the other antagonist complexes (Figures 2A and 4C). Instead, THC antagonizes ERβ by stabilizing key ligand binding pocket residues in noninteracting conformations, and disfavoring the equilibrium to the agonist-bound conformation of helix 12. Thus, we term the mechanism of antagonism of THC as "passive antagonism".

Passive antagonism may not be unique to THC and ERβ. There are many examples of NR ligands, which act as antagonists even though they are smaller than the endogenous agonists of these NRs. The synthetic androgen receptor antagonist, flutamide, is comparable in size to testosterone, and does not possess an obvious moiety that would act as an antagonist side chain[30]. Similarly, progesterone is smaller than aldosterone, yet it is a high affinity antagonist of the mineralocorticoid receptor[31,32].

Many NRs have multiple subtypes, that possess distinct expression patterns and that regulate distinct target genes **(ref)**. Antagonists generated through the addition of bulky side chains to agonist scaffolds are limited to being antagonistic on one or more subtypes of a particular NR. The passive antagonism mechanism, as revealed here through direct comparison of the two THC-ER LBD complexes,

suggests a new approach to achieving NR antagonism. Compounds could be designed to selectively stabilize the inactive conformations of certain NR subtypes and the active conformations of others. Such ligands may exert novel biological and therapeutic effects.

Methods

Transcriptional Activation Assays

The GAL4 DBD/human ERα LBD fusion expression plasmid, pDB-GAL4 ERα LBD **(ref)**, the luciferase reporter plasmid, GALRE5-e1b-TATA-Luc **(ref)**, (both provided by P. Webb), and the GRIP1 expression plasmid, pSG5 GRIP1[14], (provided by M. Stallcup) have been described. The GAL4 DBD/human ERβ LBD fusion expression plasmid (pBD-GAL4 ERβ LBD) was constructed by inserting a fragment encoding the human ERβ LBD (residues 256-505) into pBD-GAL4 (Stratagene) by standard methods.

Transient transfections were performed using an activated dendrimer transfection reagent (SuperFect; Qiagen). MDA-MB-231 breast cancer cells were seeded into 48-well tissue culture plates (Falcon, Becton Dickinson) at 1.5 x 104 cells/well in phenol red-free DMEM containing 10% heat-inactivated and charcoal-stripped fetal calf serum (DMEM-FCS; HyClone Laboratories), and incubated for 24 hrs at 37 °C in 5% CO2. Cells were washed in Dulbecco's phosphate buffered saline (DPBS) prior to transfection. The receptor expression plasmids (20 ng/well), the coactivator expression plasmid (200 ng/well), the reporter plasmid (750 ng/well), and the pRL-TK *renilla* luciferase control plasmid (Promega) (75 ng/well) were incubated with the Superfect reagent (at a ratio of 1 μg of total plasmids per 2 μL of reagent) in serum-free DMEM for 10 min at ambient temperature. The samples were then diluted six-fold in DMEM-FCS and incubated (0.3 mL/well) for 2.5 hrs at 37 °C in 5% CO2. The transfection media was removed, and the cells were washed with DPBS and incubated in DMEM-FCS with the appropriate ligand for 24 hrs at 37 °C in 5% CO2. After removal of the media, the cells were again washed in DPBS and lysed with 50 μL/well of passive lysis buffer (Promega). Samples (5 μL aliquots) were assayed for firefly luciferase and *renilla* luciferase activity using the Dual-Luciferase Reporter 1000 Assay System (25 μL each of the firefly and *renilla* luciferase substrates; Promega).

Protein Expression and Purification

The human ERα LBD (residues 297 to 554) was expressed, carboxymethylated, and purified as previously described[19]. For crystallographic studies, the human ERβ LBD (residues 256 to 505) was expressed as a N-terminally hexahistidine-tagged fusion protein in BL21(DE3)pLysS cells using the pET expression system (Novagen). Bacterial lysates were applied to an estradiol-Sepharose column[33] and the bound ERβ LBD was carboxymethylated with 20 mM iodoacetic acid[34]. Protein was eluted with 30 μM THC in ~50 ml of 20 mM Tris·Cl, 1M Urea, and 10% DMF pH 8.1. The ERβ LBD was further purified by ion exchange chromatography (Resource Q, Pharmacia). Protein samples were analyzed by SDS-PAGE, native PAGE, and electrospray ionization mass spectrometry. For biochemical studies, residues 214 to 530 of human ERβ were expressed as a fusion with glutathione-S-transferase (GST) in BL21 cells using the pGEX expression system (Pharmacia). The GST fusion protein was bound to glutathione Sepharose 4 Fast Flow (Pharmacia) and eluted with glutathione as per the manufacturer instructions. In some experiments, the protein was further purified by ion exchange chromatography (HiTrap Q, Pharmacia).

Crystallization and Data Collection

Crystals of the THC-ERα LBD-GRIP1 NR box II peptide complex were prepared by hanging drop vapor diffusion at 19-21°C . Prior to crystallization, the THC-ERα LBD complex was incubated overnight with a four-fold molar excess of the GRIP1 NR box II peptide. Samples (0.5 μL) of this solution (5.0 mg/mL protein) were mixed with 3.5 μL of reservoir buffer consisting of 16% (w/v) PEG 4000, 53 mM Tris·Cl (pH 8.8) and 50 mM $MgCl_2$ and suspended over wells containing 800 μL of the reservoir buffer. The crystals that formed lie in the spacegroup $P2_1$ with cell dimensions a=54.55 Å, b=82.60 Å, c=59.04 Å and β=111.53°. Two independent THC-LBD-peptide

complexes form the asymmetric unit. A crystal was transferred to a cryosolvent solution containing 20% (w/v) PEG 4000, 15% (w/v) ethylene glycol, 100 mM Tris·Cl (pH 8.6), 100 mM $MgCl_2$ and frozen in an N_2 stream in a nylon loop. Diffraction data were measured at -170°C at beamline 5.0.2 at the Advanced Light Source (ALS) using a Quantum 4 CCD camera (Area Detector Systems Corporation) at a wavelength of 1.10 Å. Images were processed with DENZO[35] and the integrated intensities were scaled with SCALEPACK[35] using the default -3σ cutoff.

Crystals of the THC-ERβ LBD complex were obtained by hanging drop vapor diffusion at 21-23°C. Samples (2 µL) of a solution of the complex (4.8 mg/mL) were combined with 2 µL samples of a reservoir solution containing 1.5-1.75 M $(NH_4)_2SO_4$ and 100 mM NaOAc pH 4.8-5.2 and suspended over wells containing 800 µL of reservoir solution. The resulting crystals belong to the space group R3 with cell parameters a=b=99.14 Å and c=193.38 Å (hexagonal setting). The asymmetric unit contains two ERβ LBD monomers which do not interact to form the same dimer seen in the ERα complex. Instead, each of the two LBDs interacts similarly with symmetry-related molecules to form crystallographic trimers. Prior to data collection, a single crystal was transferred to a stabilizing solution (1.8 M $(NH_4)_2SO_4$, 100 mM NaCl, 100 mM NaOAc pH 4.5, and 10 µM THC). The crystal was then sequentially transferred at 30 minute intervals through a series of solutions consisting of the stabilizing solution supplemented with increasing concentrations of ethylene glycol (1% increments) to a final concentration of 15%. The crystal was then flash frozen in an N_2 stream in a nylon loop. Diffraction data were measured at -170°C at beamline 5.0.2 at the ALS using a Quantum 4 CCD camera at a wavelength of 1.07 Å. Images were processed with DENZO and the integrated intensities were scaled with SCALEPACK using the default -3σ cutoff.

Structure Determination and Refinement

THC-ERα LBD-GRIP1 NR Box II Peptide Complex

The model of the DES-ERα LBD-GRIP1 NR Box II peptide complex (3ERD) was modified by converting the side chains of certain ligand binding pocket residues (Met 343, Leu 346, Glu 353, Leu 384, Leu 387, Met 388, Leu 391, Arg 394, Phe 404, Met 421, Ile 424, Leu 428, His 524, Leu 525, Met 528, and Leu 540) of both monomers to alanine and by removing all ligands, waters, carboxymethyl groups, and ions. After subjecting this edited model to rigid body refinement in REFMAC[36], the missing parts of the model were built, and the rest of the model was corrected using MOLOC[37] and two-fold averaged maps generated with DM[36]. All masks for averaging were generated using MAMA[38], and PHASES[39] and the CCP4 suite[36] were used for the generation of structure factors and the calculation of weights. Initially, positional refinement was performed using REFMAC. At later stages, the model was refined using the simulated annealing, positional and B-factor refinement protocols in CNS[40]. All B-factors were refined isotropically. Anisotropic scaling and a bulk solvent correction were used throughout refinement. The R_{free} set contains a random sample of 6.5% of all data. All data between 47 and 1.95 Å (with no σ cutoff) were used. The current model is composed of residues 305 to 459 and 469 to 547 of monomer A, residues 305 to 460 and 472 to 547 of monomer B, residues 687 to 696 of peptide A, residues 686 to 695 of peptide B, two ligand molecules, 167 water molecules, one carboxymethyl group, and one chloride ion. According to PROCHECK[36], **XXX** of all residues in the model are in the core regions of the Ramachandran plot, and none are in the disallowed regions.

THC-ERβ LBD Complex

The intensities within the ERβ data set fall off very rapidly as a function of resolution (Wilson B factor ~90 $Å^2$ calculated from 4.5 to 2.85 Å). In order to increase the contribution of the higher resolution terms the data were sharpened with a correction factor of –50 $Å^2$. All subsequent manipulations were performed using this sharpened data.

The two LBDs in the asymmetric unit were located by molecular replacement in AMoRe[36] and TFFC[36]. The search model was constructed by overlapping the models of five ERα LBD complexes (PDB Accession Numbers 1A52, 1ERE, 1ERR, 3ERD, and 3ERT) and setting the occupancies of each model to 20% (R= 55.3%, CC= 55.9% after placement of both monomers). The model was then rebuilt using MOLOC and two-fold averaged maps generated with DM. MAMA was used for all mask manipulations, and PHASES and the CCP4 suite were used for the generation of structure factors and the calculation of weights. Refinement was performed initially using the positional refinement protocols in REFMAC, and later using the simulated annealing, positional and B-factor refinement protocols in CNS. All B-factors were refined isotropically. Anisotropic scaling, a bulk solvent correction, and tight noncrystallographic symmetry restraints were used throughout refinement. The R_{free} set contains a random sample of 5% of all data. All data between 49 and 2.85 Å (with no σ cutoff) were used. The current model is composed of residues 261 to 284, 290 to 408, and 413 to 501 of monomer A, the last residue of the affinity tag and residues 256 to 280, 294 to 410, 413 to 479, and 485 to 497 of monomer B, two ligand molecules, and 9 water molecules. According to PROCHECK, **XXX** of all residues in the model are in the core regions of the Ramachandran plot, and none are in the disallowed regions.

Peptide Binding Assay

Samples of the GST-ERβ LBD fusion protein, in the absence or presence of the various ligands (at saturating concentrations), were mixed with an LXXLL-motif containing peptide (ILRKLLQE), which had been N-terminally labeled with rhodamine (reaction buffer = 10 mM HEPES·Na, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, and 100 μg/mL BSA at pH 7.9 and [peptide] = 5 nM). The binding reactions were incubated at ambient temperature with shaking for 1 hour in 96-well plates (Whatman), and the fluorescence polarization was measured on an Analyst reader (Molecular Devices). Receptor concentrations were determined by radioligand

binding[41]. K_d values were generated from triplicate assays by fitting the data to a simple binding isotherm (Prism, GraphPad Software).

Illustrations

Figures 2A, 2B, 4A, 4C, and 5 were generated using BOBSCRIPT[42] and rendered using Raster3D[43].

Figure Legends

Figure 1 THC Regulates ER AF-2 Activity in a Subtype-selective Manner

A. Chemical structures of 17β-estradiol (E2), ICI 164,384 (ICI), diethylstilbestrol (DES), 4-hydroxytamoxifen (OHT), and (R,R)-5,11-*cis*-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (THC). The side chains of ICI and OHT are highlighted in orange.

B. E2- and THC-mediated transcriptional response of GAL4-ER LBD chimeras in transiently transfected MDA-MB-231 breast carcinoma cells. Both chimeras activate transcription in the presence of 10 nM E2, but only the ERα chimera activates in the presence of 1 μM THC. Values represent the mean ± SD for each experimental condition performed in triplicate.

Figure 2 Overall Structures of the THC-ER LBD Complexes

A. Two equivalent orthogonal views of the THC-ERα LBD-GRIP1 NR box II peptide and the THC-ERβ LBD complexes, showing that the two ER LBDs adopt distinct conformations when bound to THC. Both the ERα and ERβ LBDs are depicted in ribbon representation, and are colored light green and light blue, respectively. In both complexes, helix 12 is colored red, and THC, shown in spacefilling representation, is colored green. In the ERα complex, the coactivator peptide is depicted as a purple ribbon.

B. Superpositions of the Cα trace of the THC-ERβ LBD complex (cyan) on those of the RAL-ERβ LBD (orange) and the GEN-ERβ LBD complexes (yellow). Helix 12 in the RAL and GEN structures is colored red. Least squares superpositions were generated using LSQMAN[44] (THC/RAL: 0.95 Å r.m.s.d. over 196 matched atoms with a 3.8 Å cutoff, THC/GEN: 1.06 Å r.m.s.d. over 218 matched atoms with a 3.8 Å cutoff).

Figure 3 Ligands Perturb the Equilibrium Between Two Distinct Conformations of Helix 12

A. Schematic representation of the effects of different classes of ligands on the described helix 12 conformational equilibrium. Helix 12, colored red, is depicted in two conformations: the "inactive" apo/THC-or GEN-bound conformation and the "active" agonist-bound conformation. The inactive conformation prevents coactivator association, and the active one favors it.

B. Equilibrium binding of a rhodamine-labeled LXXLL-motif containing peptide to the ERβ LBD alone or bound to E2, DES, GEN, and THC was analyzed by measuring fluorescence polarization as a function of receptor or ligand-receptor complex concentration. Values represent the mean ± SD for each experimental condition performed in triplicate and the data were fit to a simple binding isotherm.

Figure 4 THC Interactions with the ERα and ERβ Ligand Binding Pockets

A. $2F_o$-F_c electron density of THC in the ERα complex (green) and in the ERβ complex (orange). For the ERα complex, the map was calculated to 1.95 Å and contoured at 1.2σ, and for the ERβ complex, the map was calculated to 2.85 Å and contoured at 1.0σ. Both maps were calculated after omitting the ligand. The ethyl groups of THC are rotated under the tetrahydrochrysene scaffold when bound to ERα, but are rotated outwards when bound to ERβ.

B. Schematic diagrams of the interactions between THC and the binding pockets of the two ERs. Only residues or waters that form hydrogen bonds with THC and/or van der Waals contacts with THC (4.2 Å cutoff) are depicted. Hydrogen bonds and van der Waals contacts are represented by cyan lines and gray lines, respectively. Hydrogen bond distances (in Å) are given. Residues that only interact with THC in the ERα complex are green and those that only interact in the ERβ complex are blue. The A and A' rings and the individual atoms of THC are labeled.

C. The structures of the two THC-ER complexes were superimposed using LSQMAN (1.13 Å r.m.s.d. over 200 matched Cα atoms using a 3.8 Å cutoff). ERα and

ERβ residues are labeled in light green and light blue, respectively. Side chain atoms are colored by atom type (carbon(ERα)=light green, carbon(ERβ)=light blue, nitrogen=dark blue, oxygen=red, sulfur=yellow). THC in the ERα complex is colored green and THC in the ERβ complex is colored orange. Hydrogen bonds are depicted as dashed dark gray bonds. For clarity, water molecules and the side chains of Thr 347 and Leu 349 from ERα and Thr 299 and Leu 301 from ERβ are not shown.

Figure 5 Model of THC-ERβ LBD in the Agonist-Bound Conformation

A model of THC-ERβ LBD complex in the agonist-bound conformation was generated by overlapping the A ring of THC with that of GEN in the GEN-ERβ complex and optimally positioning the THC scaffold between the side chains of Met 336 and Ile 373. This model and the structure of the THC-ERβ complexes were superimposed on that of the THC-ERα complex using LSQMAN. For the two crystallographically-determined THC-ER complexes, side chain atoms and THC are colored as in Figure 4C. For the modeled complex, THC is colored red, carbon side chains are colored magenta, and sulfur side chain atoms are colored yellow. Semi-transparent van der Waals surfaces for one of the THC ethyl groups and the side chain of Leu 298 are drawn to illustrate the unfavorable close contacts in the model.

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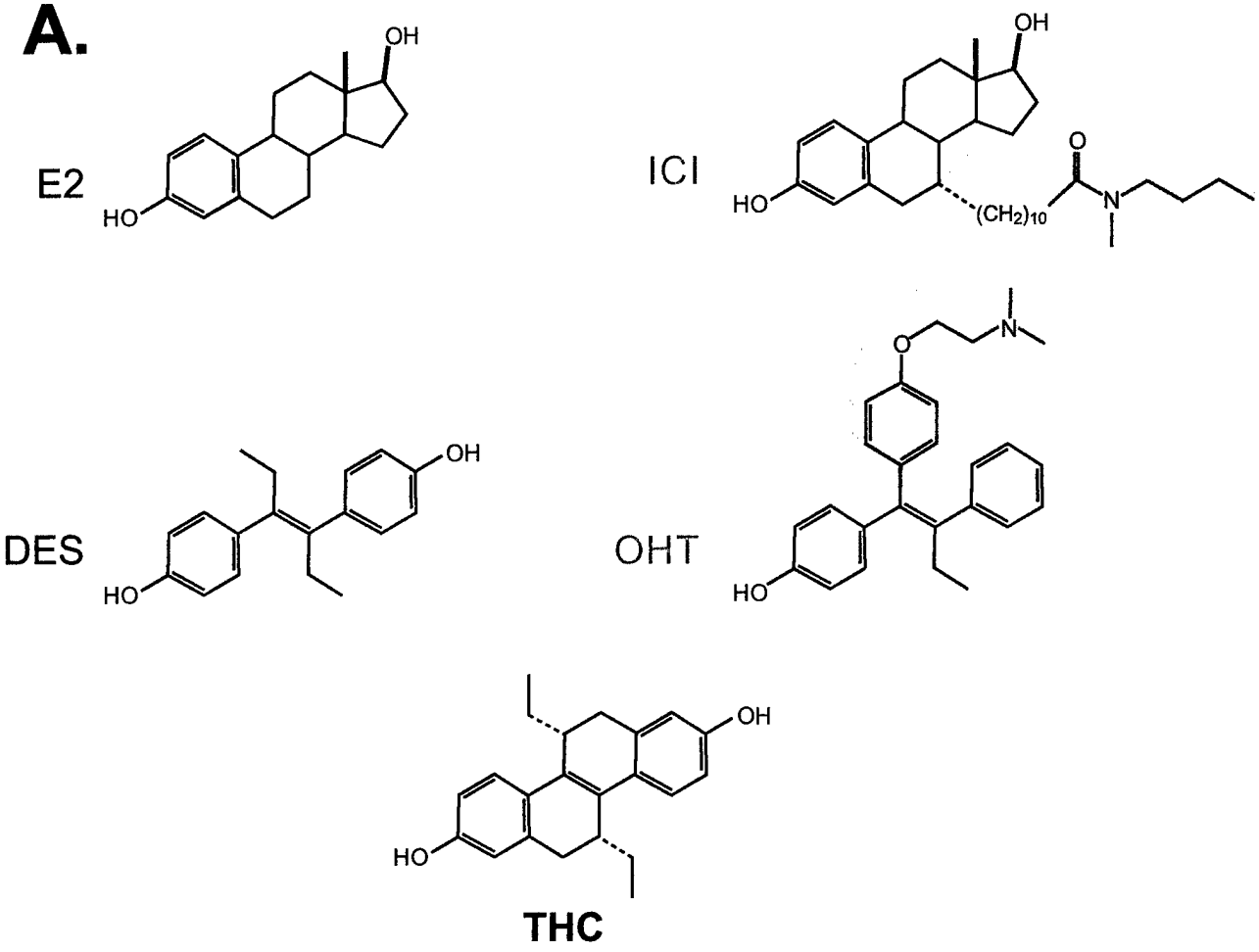
Table 1: Summary of Crystallographic Statistics

Data Collection		
Complex	THC-ERα LBD-GRIP1 NR Box II Peptide	THC-ERβ LBD
Space group	$P2_1$	R3
Resolution	1.95	2.85
Observations	119409	71300
Unique	34533	16522
Completeness (%)	97.9	99.7
R_{sym} (%)[a]	6.5	4.7
Average I/σI	12.3	14.8
Refinement		
Non-hydrogen protein atoms	3830	3409
Water molecules	167	9
Non-hydrogen heterogen atoms	53	48
R_{cryst} (%)[b]/R_{free} (%)	20.3/24.2	26.5/30.4
Bond r.m.s. deviation (Å)	0.005	0.011
Angle r.m.s. deviation (°)	1.08	1.29
Average B factor (Å^2)	38.5	40.1

[a] $R_{sym} = \Sigma | I_h - \langle I_h \rangle | / \Sigma I_h$, where $\langle I_h \rangle$ is the average intensity over symmetry equivalents.

[b] $R_{cryst} = \Sigma | F_o - F_c | / \Sigma | F_o |$, where F_o and F_c are observed and calculated amplitudes, respectively.


A.
E2
OH
HO
ICI
OH
HO
O
N
(CH2)10
DES
OH
HO
OHT
O
N
HO
OH
HO
THC

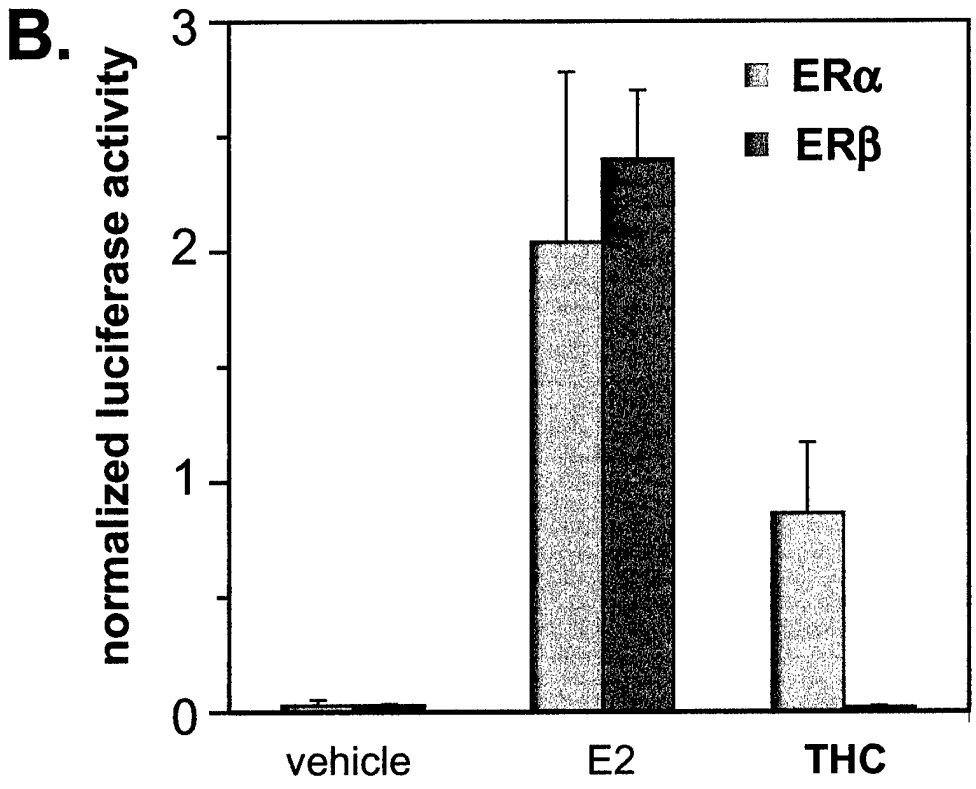

B.
normalized luciferase activity
3
2
1
0
ERα
ERβ
vehicle
E2
THC

Figure 1

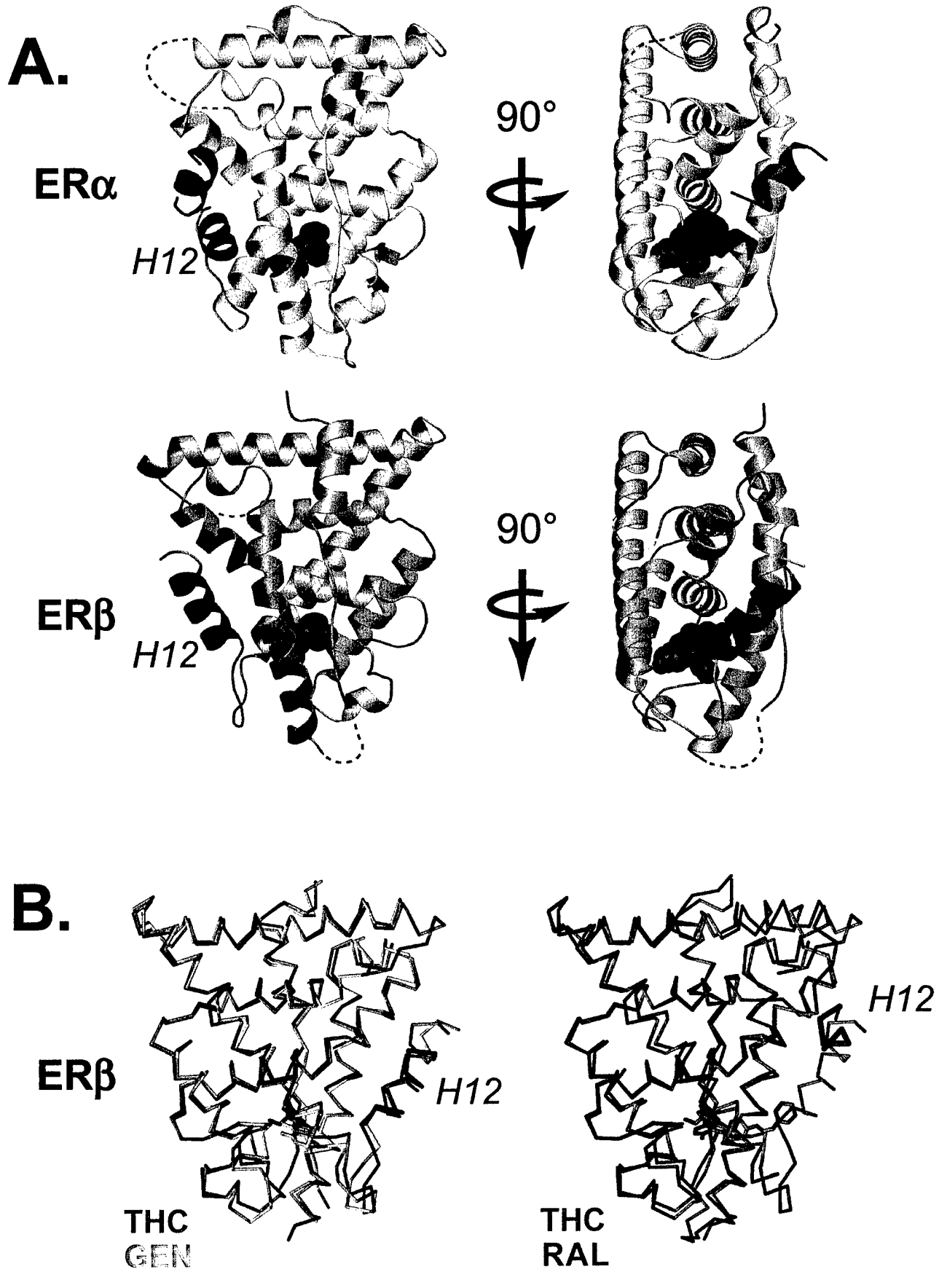
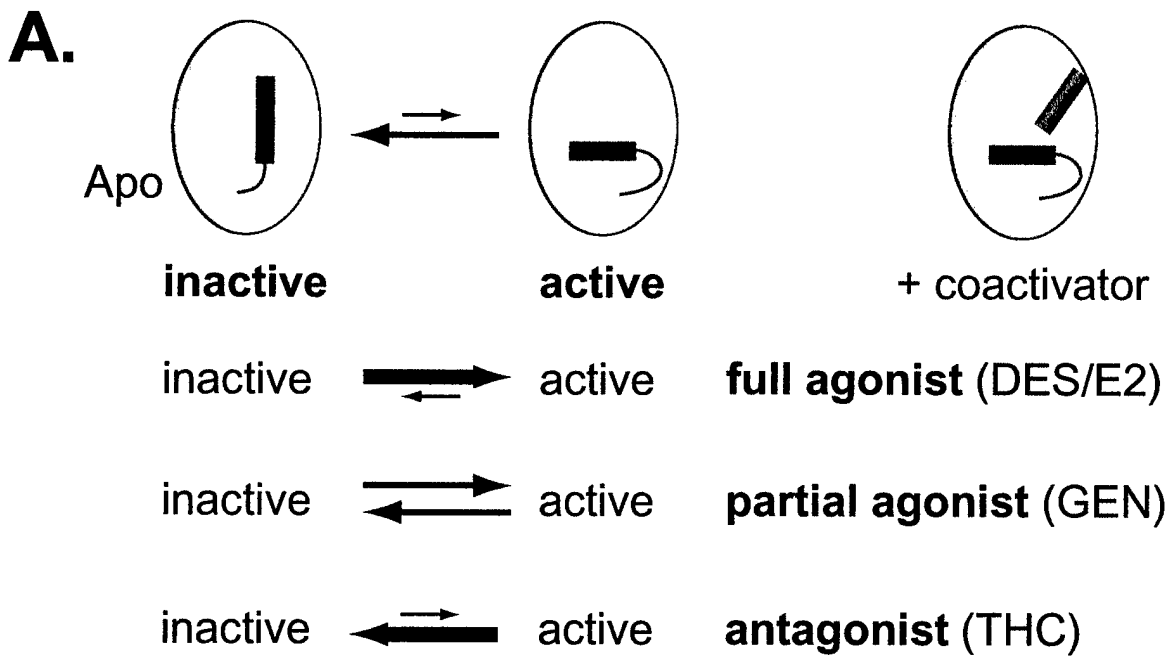


Figure 2


A.
Apo
inactive
active
+ coactivator
inactive
active
full agonist (DES/E2)
inactive
active
partial agonist (GEN)
inactive
active
antagonist (THC)

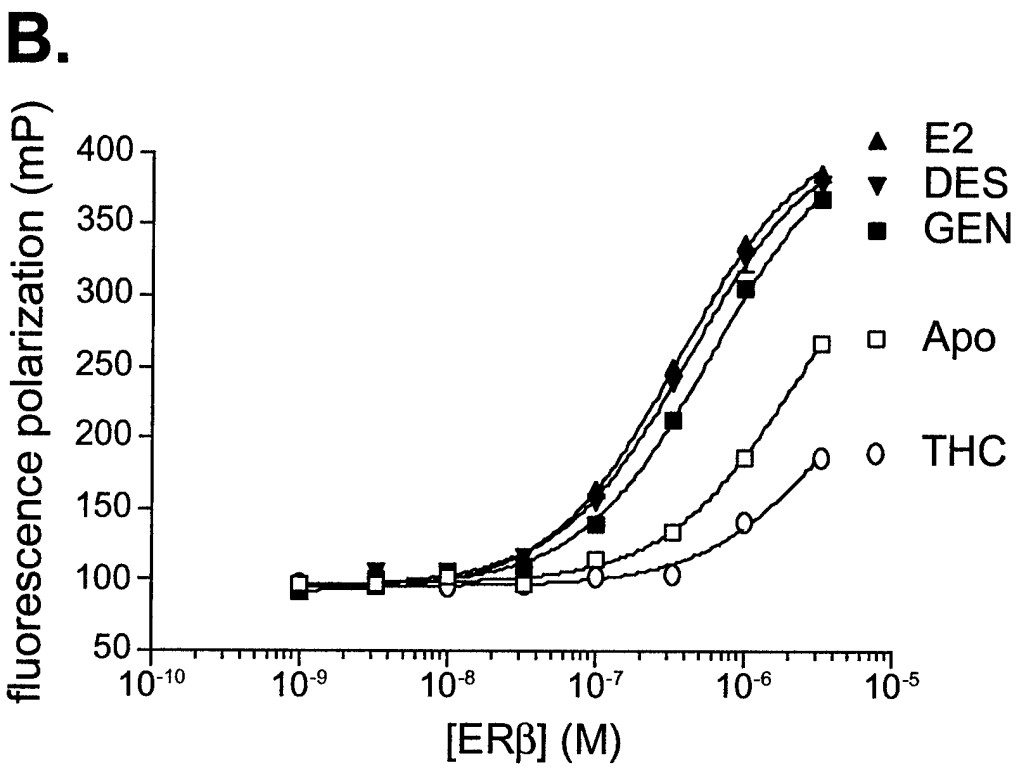

B.
fluorescence polarization (mP)
400
350
300
250
200
150
100
50
10-10
10-9
10-8
10-7
10-6
10-5
[ERβ] (M)
E2
DES
GEN
Apo
THC

Figure 3

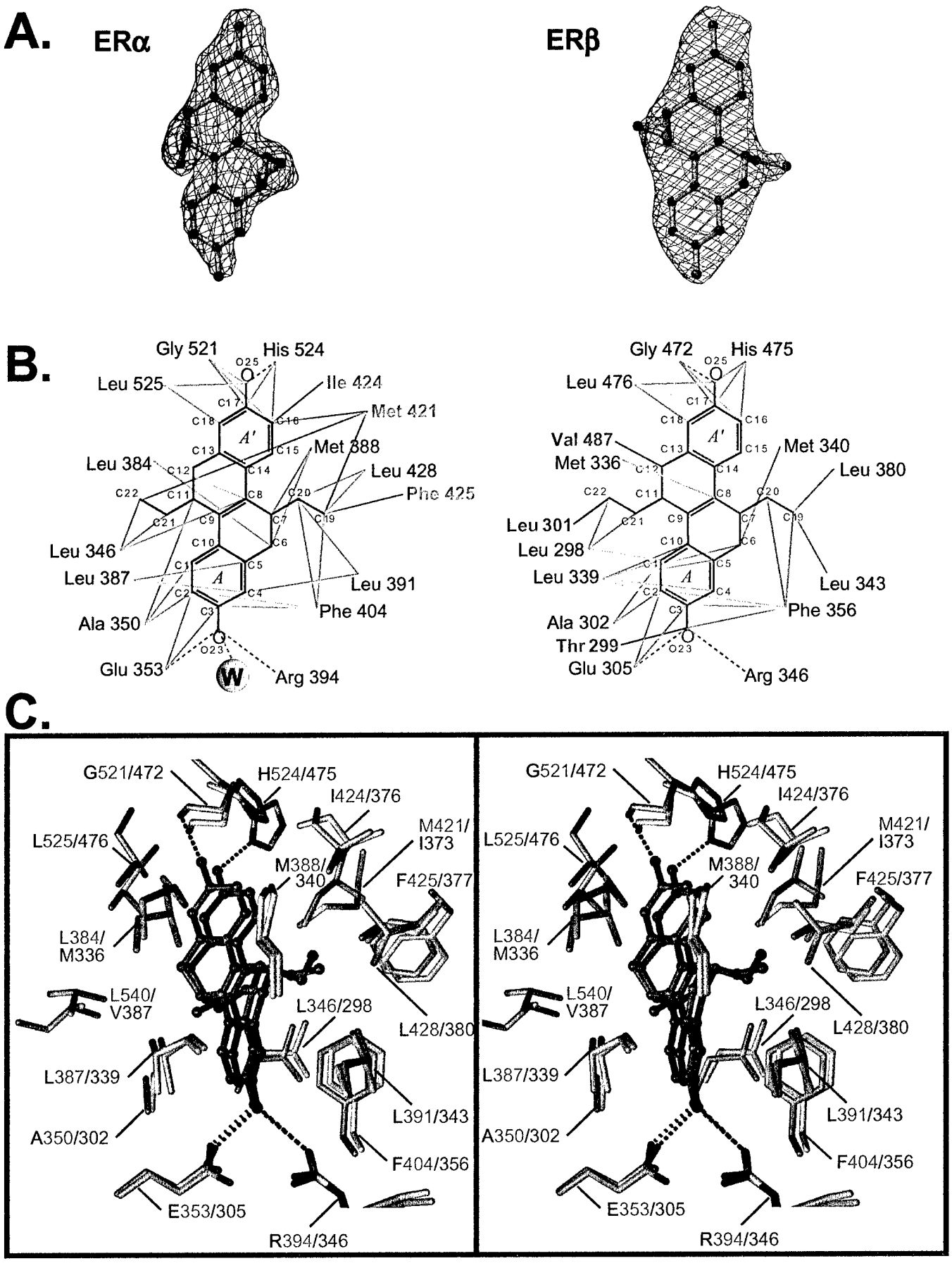

A.
ERα
ERβ
B.
Gly 521
His 524
Leu 525
Ile 424
Met 421
Met 388
Leu 384
Leu 428
Phe 425
Leu 346
Leu 387
Leu 391
Phe 404
Ala 350
Glu 353
Arg 394
Gly 472
His 475
Leu 476
Val 487
Met 336
Met 340
Leu 380
Leu 301
Leu 298
Leu 339
Leu 343
Phe 356
Ala 302
Thr 299
Glu 305
Arg 346
C.
G521/472
H524/475
I424/376
L525/476
M421/
I373
M388/
340
F425/377
L384/
M336
L540/
V387
L346/298
L428/380
L387/339
L391/343
A350/302
F404/356
E353/305
R394/346

Figure 4

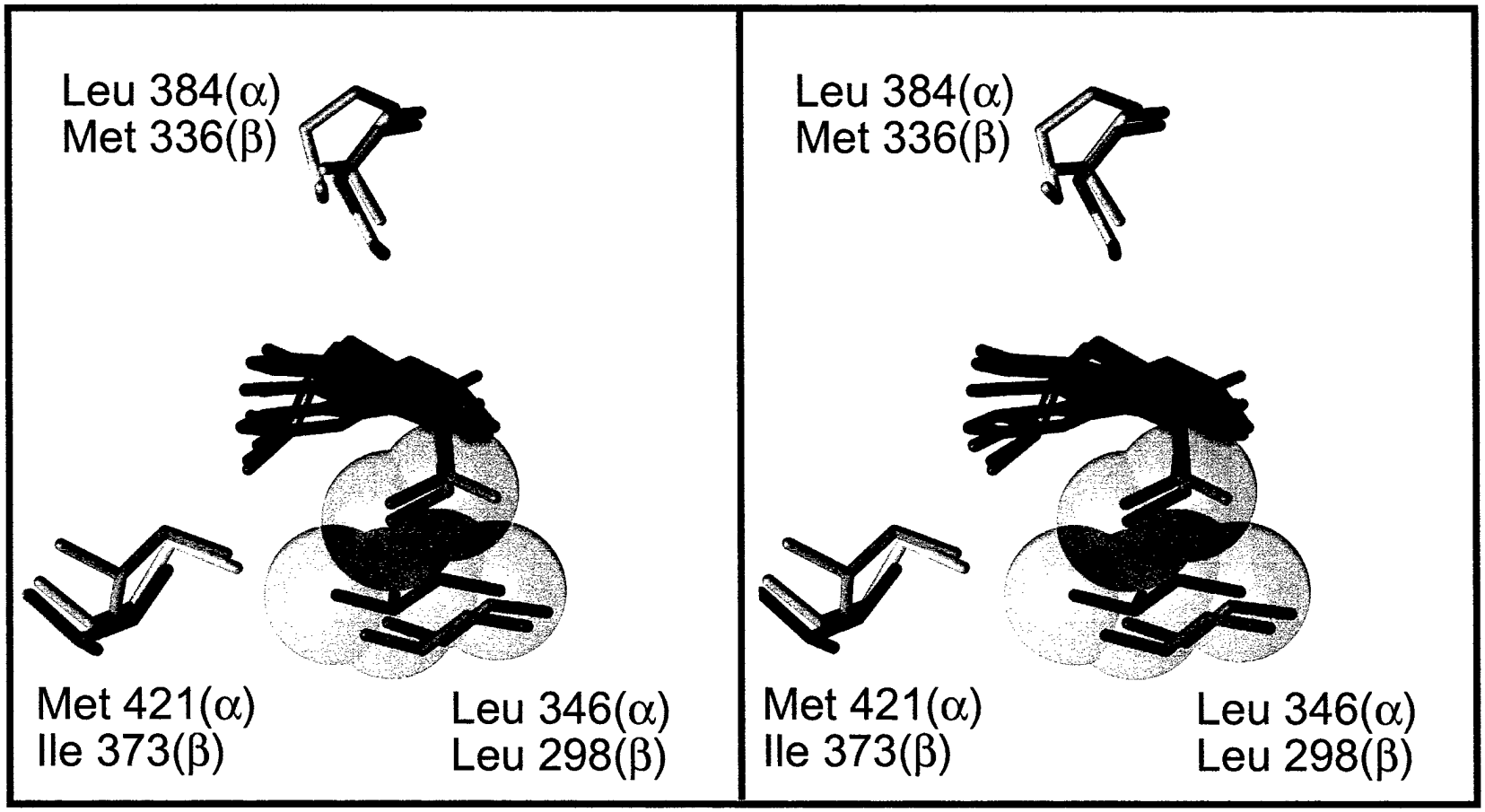

Leu 384(α)
Met 336(β)
Met 421(α)
Ile 373(β)
Leu 346(α)
Leu 298(β)
Leu 384(α)
Met 336(β)
Met 421(α)
Ile 373(β)
Leu 346(α)
Leu 298(β)

Figure 5

01-L

STRUCTURAL AND MECHANISTIC ASPECTS OF SERM ACTION MEDIATED BY ERα AND ERβ.

G. L. Greene,[+] A. Shiau,[*] D. Barstad,[+] P. Kushner,[±] and D. Agard[*]. [+]Ben May Institute for Cancer Research and Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois 60637; [*]Howard Hughes Medical Institute and the Department of Biochemistry & Biophysics, University of California at San Francisco; [±]Metabolic Research Unit, University of California at San Francisco, San Francisco, CA 94143, U.S.A.

Estrogens and estrogen antagonists induce characteristic conformational changes in the estrogen receptor (ERα/β) that influence dimerization, phosphorylation, interaction with general and specific effector molecules, recognition of specific DNA response elements in target genes, and modulation of transcriptional activity. Many of these processes are tissue- and promoter-specific, which can change the balance between the agonistic or antagonistic behavior of a given ligand. Tamoxifen and raloxifene represent a growing class of diverse molecules known as selective estrogen receptor modulators (SERMs), which can be distinguished from pure estrogens by their ability to act either as estrogens (agonist) or as antiestrogens (antagonist) in different tissues, gene contexts and hormone environments. To better understand this selective behavior, the structures of the human ERα ligand binding domain (ERα-LBD) complexed with several estrogen agonists and antagonists were determined. Insight into the molecular basis of estrogen agonism and antagonism has been revealed by the crystal structures of ERα and ERβ ligand binding domains (LBDs) complexed with several ligands, including estradiol (E2), diethylstilbestrol (DES), raloxifene (RAL), 4-hydroxy-tamoxifen (OHT), and genistein (GEN). For agonists like DES, inclusion of a peptide derived from an essential LXXLL interaction motif (NR box) found in several related p160 nuclear receptor transcriptional co-activators has helped define the AF-2/co-activator interface. Although agonists and antagonists bind at the same site within the core of the LBD, each induces distinct conformations in the transactivation domain (AF-2) of the LBD, especially in the positioning of helix 12, providing structural evidence for multiple mechanisms of selective antagonism in the nuclear receptor family. Interestingly, the OHT/RAL and DES/E2 structures collectively reveal and define a multipurpose docking site on ERα that can accommodate either helix 12 or one of several co-regulators. In addition, a comparison of the two structures reveals that there are at least two distinct mechanisms by which structural features of OHT promote an "autoinhibitory" helix 12 conformation. Helix 12 positioning can be determined both by steric considerations, such as the presence of an extended side chain in the ligand, and by local structural distortions in and around the ligand binding pocket. Thus, one would predict that effective estrogen antagonists do not necessarily require bulky or extended side chains. It is anticipated that the utilization of subtype-specific interactions in ligand design will allow the creation of new compounds that act differently on the two ERs and possess novel therapeutic properties. In addition, with the design and/or natural occurrence of compounds that selectively target ERα or ERβ, structure information should help reveal the molecular basis for such behavior.

Correlations Between ERα/β Structure and SERM Behavior

Geoffrey L. Greene, PhD

Insight into the molecular basis of estrogen agonism and antagonism has been revealed by the crystal structures of ERα and ERβ ligand binding domains (LBDs) complexed with several ligands, including estradiol (E2), diethylstilbestrol (DES), raloxifene (RAL), 4-hydroxy-tamoxifen (OHT), and genistein (GEN). For agonists like DES, inclusion of a peptide derived from an essential LXXLL interaction motif (NR box) found in several related p160 nuclear receptor transcriptional coactivators has helped define the AF-2/co-activator interface. Although agonists and antagonists bind at the same site within the core of the LBD, each induces distinct conformations in the transactivation domain (AF-2) of the LBD, especially in the positioning of helix 12, providing structural evidence for multiple mechanisms of selective antagonism in the nuclear receptor family. Interestingly, the OHT/RAL and DES/E2 structures collectively reveal and define a multipurpose docking site on ERα that can accommodate either helix 12 or one of several coregulators. In addition, a comparison of the two structures reveals that there are at least two distinct mechanisms by which structural features of OHT promote an "autoinhibitory" helix 12 conformation. Helix 12 positioning can be determined both by steric considerations, such as the presence of an extended side chain in the ligand, and by local structural distortions in and around the ligand binding pocket. Thus, one would predict that effective estrogen antagonists do not necessarily require bulky or extended side chains.

The crystallographic structures of *cis*-R,R-diethyl-tetrahydrochrysene (R,R-THC) bound to the ligand binding domains of ERα and ERβ have recently been determined, suggesting mechanisms by which this compound can act as an ERα agonist and as an ERβ antagonist. Consistent with the prediction that bulky/extended side chains are not essential for antagonist behavior, R,R-THC antagonizes ERβ in a manner very different to OHT and

Lessons learned from the structures of ERα and ERβ bound to SERMs

Greene GL* (1), Shiau AK (2), Barstad D (1), Katzenellenbogen JA (3) Katzenellenbogen BS (3) and Agard DA (4).
(1) The Ben May Institute for Cancer Research, University of Chicago, Chicago, Illinois 60637 (2) Tularik Inc, South San Francisco, California 94080 (3) Dept. of Chemistry and Dept. of Molecular and Integrative Physiology, University of Illinois, Urbana, Illinois 61801. (4) Howard Hughes Medical Institute and Dept. of Biochemistry & Biophysics, University of California, San Francisco, CA 94143.

Insight into the molecular basis of estrogen agonism and antagonism has been revealed by the crystal structures of ERα and ERβ ligand binding domains (LBDs) complexed with several ligands, including estradiol (E_2), diethylstilbestrol (DES), raloxifene (RAL), 4-hydroxy-tamoxifen (OHT), and genistein (GEN). For agonists like DES, inclusion of a peptide derived from an essential LXXLL interaction motif (NR box) found in several related p160 nuclear receptor transcriptional co-activators has helped define the AF-2/co-activator interface. Although agonists and antagonists bind at the same site within the core of the LBD, each induces distinct conformations in the transactivation domain (AF-2) of the LBD, especially in the positioning of helix 12, providing structural evidence for multiple mechanisms of selective antagonism in the nuclear receptor family. Interestingly, the OHT/RAL and DES/ E_2 structures collectively reveal and define a multipurpose docking site on ERα that can accommodate either helix 12 or one of several coregulators. In addition, a comparison of the two structures reveals that there are at least two distinct mechanisms by which structural features of OHT promote an "autoinhibitory" helix 12 conformation. Helix 12 positioning can be determined both by steric considerations, such as the presence of an extended side chain in the ligand, and by local structural distortions in and around the ligand binding pocket. Thus, one would predict that effective estrogen antagonists do not necessarily require bulky or extended side chains.

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Molecular Basis of Estrogen Responsiveness

Geoffrey L. Greene. The Ben May Institute for Cancer Research, University of Chicago, Chicago, Illinois 60637

Estrogens and SERMs (selective estrogen receptor modulators) regulate diverse cellular activities via one or both of two known estrogen receptor subtypes (ERα and ERβ) in hormone responsive tissues and cancers. Liganded ERs can interact with a complex mix of coactivators, corepressors and other signaling molecules that differ in expression and importance from tissue to tissue. In addition, different SERMs may alter the affinity and/or selectivity of one or both ERs for these coregulators, allowing for tissue selective responses. Recently, insight into the molecular basis of estrogen agonism and antagonism has been revealed by the crystal structures of ERα and ERβ ligand binding domains (LBDs) complexed with several ligands, including estradiol (E2), diethylstilbestrol (DES), raloxifene (RAL), 4-hydroxytamoxifen (OHT), and the phytoestrogen genistein (GEN). For agonists like DES, inclusion of a peptide derived from an essential LXXLL interaction motif (NR box) found in several related p160 nuclear receptor transcriptional co-activators has helped define the AF-2/co-activator interface on ER. Although agonists and antagonists bind at the same site within the core of the LBD, each induces distinct conformations in the transactivation domain (AF-2) of the LBD, especially in the positioning of helix 12, providing structural evidence for multiple mechanisms of selective antagonism in the nuclear receptor family. Interestingly, the OHT/RAL and DES/E2 structures collectively reveal and define a multipurpose docking site on ERα that can accommodate either helix 12 or one of several coregulators. In addition, a comparison of the two structures reveals that there are at least two distinct mechanisms by which structural features of OHT promote an inhibitory conformation of helix 12. Helix 12 positioning is determined both by steric considerations, such as the presence of an extended side chain in the ligand, and by local structural distortions in and around the ligand binding pocket. Thus, one would predict that effective estrogen antagonists do not necessarily require bulky or extended side chains.

The crystallographic structures of a novel synthetic SERM, *cis*-R, R-diethyl-dihydroxy-tetrahydrochrysene (R,R-THC), bound to the ligand binding domains of ERα and ERβ have recently been determined, suggesting mechanisms by which this mixed activity ligand can function as an ERα agonist and as an ERβ antagonist. Consistent with the prediction that bulky/extended side chains are not essential for antagonist behavior, R,R-THC antagonizes ERβ in a manner very different from OHT and RAL, by filling the ligand-binding pocket of ERβ sub-optimally and acting as a passive antagonist. It is anticipated that the utilization of ER subtype-specific interactions in ligand design will allow the creation of new compounds that act differently on the two ERs and possess novel therapeutic properties. In addition, with the design and/or natural occurrence of compounds that selectively target ERα or ERβ, structure information should help reveal the molecular basis for such behavior. Ultimately, this information must be coupled with information about expression patterns for both ER subtypes as well as essential coregulatory proteins or intersecting signal pathways to predict the behavior of a given SERM in a responsive tissue.

EMBO Workshop on Nuclear Receptor Structure and Function,
Erice, Sicily, Italy, 5/01

Structure/function analyses of SERMs complexed with ERα and ERβ: mechanistic implications

Geoffrey L. Greene. The Ben May Institute for Cancer Research, University of Chicago, Chicago, Illinois 60637

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